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Advances in Marine Environmental Research

Proceedings of a Symposium

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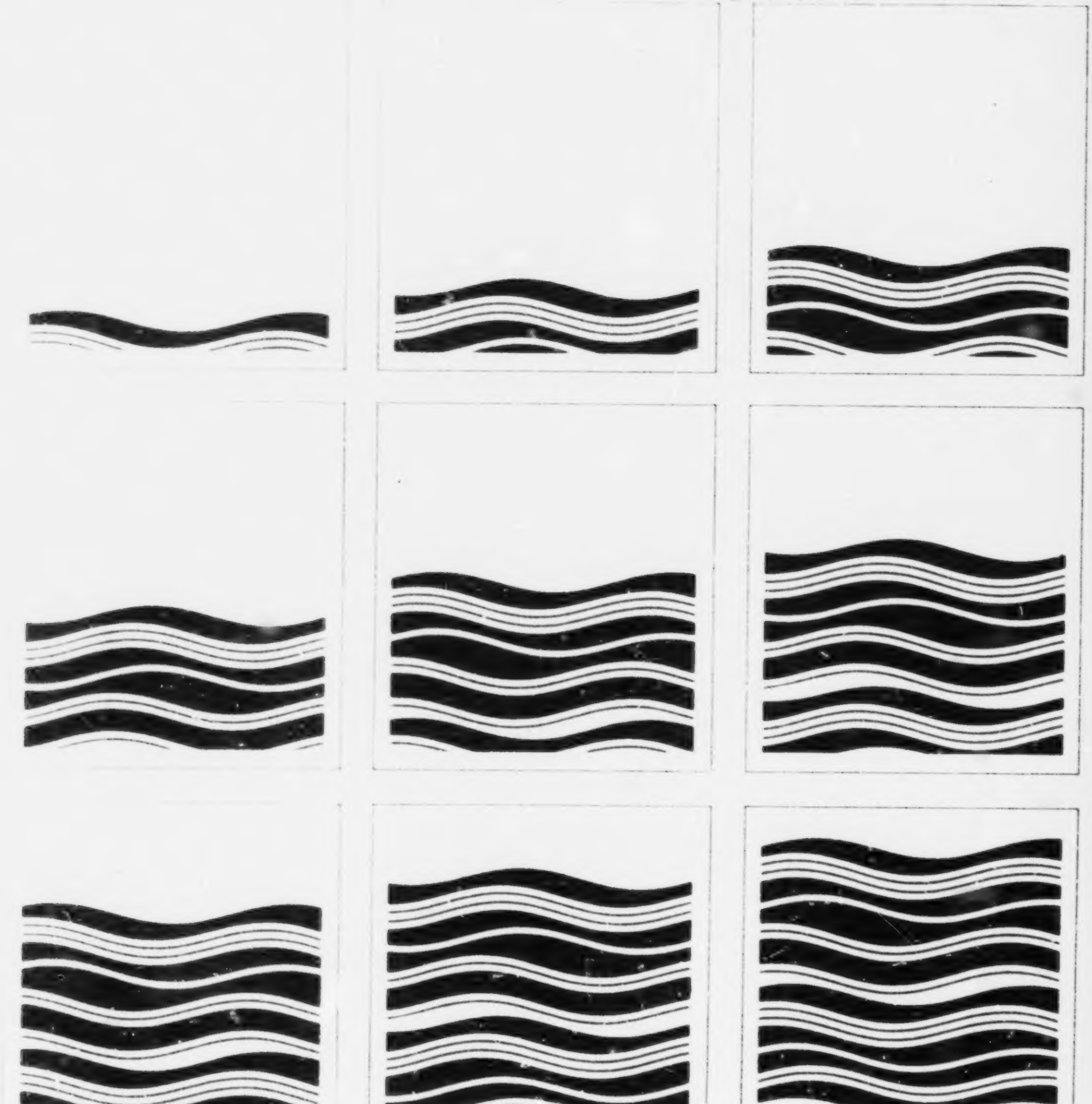

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September 1979

Advances in Marine Environmental Research

Proceedings of a Symposium

Francine Sakin Jacoff,
Editor

Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Narragansett, Rhode Island 02882

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DEDICATION

ADVANCES IN MARINE ENVIRONMENTAL RESEARCH

A decade ago, there were only a handful of scientists throughout the world engaged in the field of science called ecology. With rising social consciousness and an escalating series of local, national and global environmental problems, there was an outcry for the application of scientific analysis to these problems. The result of this was an evolution of a new field, called pollution research, which had as its cornerstone the science of ecology.

This volume includes papers that will discuss many of the specific aspects of marine ecology and marine pollution research. You will find authors who are studying the transformation and movements of pollutants in chemical systems, as well as those who are attempting to miniaturize and model ecosystems with the microcosms. The papers contained herein are a benchmark of marine pollution research.

We are dedicating the volume to one of the founders of modern ecology and marine pollution research, Eugene P. Odum. Dr. Odum has dedicated his life to understanding the holistic processes of ecosystems and man's interaction with these complex biological, physical and chemical systems. His pioneering work in wetlands and radioecology led to his synthesized works in FUNDAMENTALS OF ECOLOGY. The more that we attempt to understand and unravel the complexities of modern marine ecosystems, the more we recognize that the basic principles espouse by Eugene Odom are true. Not only are we realizing that we cannot uncouple the various components of ecosystems, but that man himself is coupled into these complex systems.

Do not read the volume with the expectation of understanding all the answers to major marine pollution problems today–but read it as a state-of-the-art document outlining our advances in a rapidly changing and evolving science. Throughout all the papers, attempt to follow Odum's guidance, and to understand how the discussion of various parts of the problem can be combined into holistic concepts that have eluded us in the past. Our field of marine ecology has not evolved to that of a predictive science; we lack basic hypotheses and understandings to make it so.

I hope that the reader will view these papers through Odum's "macroscope," and in this way gain insights into the holistic view of our oceans and coastal waters that will allow man to live in closer harmony with the sea.

Eric D. Schneider, Director
Environmental Research Laboratory
Narragansett, R.I.

KEYNOTE ADDRESS

DEDICATION OF NEW WING OF NARRAGANSETT EPA LABORATORY JUNE 1977

Delivered by
Eugene P. Odum, Director
Institute of Ecology, University of Georgia

The theme of my address at today's dedication is that the time has come to adopt a holistic approach to researching and managing our environmental problems. This is not to say that we abandon the traditional reductionist way of science which involves dividing up a complex problem into small components that are then assigned to specialists for detailed study. Rather, we perhaps need to follow the general procedure we use in microscopy, namely, shifting back and forth between powers so as to examine the subject at different levels of organization. To put it another way, we need to develop the "macroscope" as a tool as well as the microscope. Most of all, we need to promote integrated team research as well as reward the individual effort that is the traditional, and too often the only, criterion for promotion in universities and research institutions.

Reductionism in science has led to important discoveries in physics, chemistry, molecular biology and genetics, but this approach comes up short in ecology where the exciting problems, and also those of most concern to society, lie at the ecosystem level rather than at the molecular level. The Environmental Protection Agency was organized by society to fight cancer at the ecosystem level, not at the cell or organism level. Theories, and tools, must be organized accordingly, since procedures appropriate for one level of concern may not be appropriate at all for another level of study.

Holism as a basic operational principle or paradigm rests on the theory of hierarchal systems, a theory not yet fully understood nor accepted by many scientists. Since there is both continuity and discontinuity in the evolution of the universe, development may be viewed as continuous because it is never-ending, but also discontinuous because it passes through a series of different levels of organization with vertical as well as horizontal integration. The keystone in the theory of hierarchal organization is the concept of emergent properties. As components, or subsets, are combined to produce larger functional wholes, new properties emerge that were not present or not evident at the next level below. In speaking of these matters in general lectures, I often use water as an example. Water has many unique properties not shared by the components, hydrogen and oxygen. To cite a few, it is a liquid, chemically inert, and has its maximum density at 4°C; in contrast to the two gaseous components having none of these characteristics. It is obvious that the holistic approach of studying water as water (as a whole molecular complex) would reveal these important integrative, or "emergent", properties more easily and quickly than the reductionist approach keying on the study of the component parts. Thus, it would be very difficult, if not impossible, to deduce the maximum-density-at-4^{o}C property of water from knowledge of the properties of hydrogen and oxygen as they occur in their separate states.

Thus, the forest is indeed more than a collection of trees, to quote an old adage. As a specific example of emergent properties at the ecosystem level, I might cite the work my brother and I did on a coral reef on a Pacific Atoll, as was alluded to by Frank Lowman in his introduction. We measured the metabolism of the intact reef by monitoring oxygen changes in the water flowing over the reef. We also did a detailed trophic analysis as a means of charting major energy flows, and were able to construct an energy budget for the whole system. It became evident from these analyses that coral animals and associated algae were much more closely linked metabolically than had previously been supposed, and that the inflow of nutrients and animal food from surrounding ocean water was inadequate to support the reef community if corals and other biota were functioning in ordinary food chains. We theorized that the observed very high rate of productivity for the reef as a whole was an emergent property resulting from symbiotic linkages that maintain efficient energy use and nutrient recycling between autotrophic and heterotrophic components. I believe we can say that subsequent work on Pacific reefs has verified this hypothesis.

As an interesting aside, we suggest that these coral reef discoveries have at least philosophical significance for urban-industrial man. The Pacific coral reef as an oasis in a desert ocean can stand as an object lesson for man who must now realize that mutalism between autotrophic and heterotrophic components, and between producers and consumers in the societal realm, coupled with efficient recycling of materials and use of energy, are the keys to maintaining prosperity in a world of limited resources. Only by moving up in our thinking, in our research, and in our management to the ecosystem level in the hierarchal system can we accomplish this vital mission. During the industrial revolution mankind essentially "uncoupled" himself from nature. Because the individual in industrial societies no longer is directly dependent on the natural environment for his day-to-day needs, he forgets how dependent we really are on natural processes that produce food, recycle water, purify air, and so on. Our food, for example, comes in on a long and complex chain of production, processing, and transportation so we are not really aware of where it came from or how much energy was expended, or how much pollution created, and so on. It is definitely time to recouple the house of man and the house of nature and assess and manage them as one integrated ecosystem.

In recent months the writing of environmental impact statements, as required by NEPA, has been criticized in the pages of *Science* and other professional magazines as being superficial and exercises of bureaucratic futility. As I see it, current impact assessment is not so much bad or inadequate science as it is *wrong-level* applied science, a viewpoint that has not been emphasized in recent discussions of the subject. In other words, if NEPA is to survive the economic and political pressure of the future, assessment must evolve as rapidly as possible from the present largely descriptive component approach to a more holistic approach which combines the use of broad ecosystem-level indices of structure and function with specific local or population factors (i.e., "red flags") that are of special public concern (such as fish or game, or an endangered species). Also, economic and ecologic considerations must be integrated, not undertaken as separate studies without common denominators. This can be done, and if I had time I could describe two cases where we were successful in such a merger. (Write me and I'll send reprints.)

Finally, the impact-assessor and the decision-maker should be part of the same team, or at least sit around the same table to review all the alternatives. In other words, a good assessment cannot be made piecemeal any more than one can understand water or a coral reef by component study alone.

So much for general theory; now for some suggestions for EPA and Directors of EPA laboratories. In pursuing its mission to reduce and control pollution, EPA has so far concentrated efforts in two areas: (1) monitoring technology, designed to determine the what, where, and how much of undesirable inputs into our environment, and (2) control technology and regulations designed to roll back the tide of effluents which threaten our health and the quality of our life. These efforts, of course, are appropriate and need to be continued without let-up, but they are essentially negative in approach since they indicate to industry and to people in general what they must not do, but not what they can do. I believe the time has come to add two positive dimensions to the menu; namely, (1) waste-management systems that couple in-house waste treatment with the assimilatory capacity of surrounding natural ecosystems that serve as the ultimate tertiary treatment plants, and (2) a merging of ecologic and economic assessments, along lines mentioned in my earlier review of theory so as to demonstrate what we all believe to be true; namely, that the economic return of clean environments is greater than the short-term gains that may result from ignoring or postponing pollution abatement.

As an example of the first of these suggested new research areas, I can cite a project that we at the University of Georgia have undertaken under contract with a large industrial company. In this case the company proposes to build a chemical plant on a site that is adjacent to an extensive area of natural wetlands, both swamp forests and marshes, which we believe have considerable capacity to assimilate and recycle nutrients and bio-degradable wastes. By both inventory and experimental procedures we are in the process of determining just what this "tertiary" treatment capacity is with the understanding that the company will then design their in-house treatment facilities so as to remove the toxic substances and release into the wetland environment only that which can be assimilated. Such a procedure I like to call "reciprocal design" in that both the industrial engineer and the ecologist have the same objective; namely, essentially zero pollution after effluents have passed through both the man-made and the natural filters. In this case, the company owns the wetlands which, when used in the manner described, become a highly valued part of a total waste management system. I believe there would be much to be gained if EPA laboratories entered into "reciprocal design" contracts with industry, and thus become partners, rather than adversaries in the pursuit of common goals.

Merging economics and ecology may prove difficult, but it does make common sense since the two words have a common Greek root, "oikos" meaning "household"; ecology literally is "the study of the household," and economics "the management of the household." The trouble is that "nature's house" is entirely external to "man's house" in current economic procedures, so that the very valuable and necessary work of nature, such as the tertiary treatment of wastes just discussed, is not included in economic cost-accounting or in the workings of the market system. In discussing theory, I made a point of the need to recouple the "houses" of man and nature, so we can follow up by suggesting that the best practical way to do this is to find ways to internalize into the economic system what are now considered to be the "free goods and services" of nature.

I will close by mentioning several special marine research challenges, since this laboratory focuses on coastal and marine environments. Microbial components and transformations in marine and estuarine environments are the least known, yet the most important aspects when it comes to systems metabolism and the impact of man-made perturbations. Microbial activities in the anaerobic layers of sediments and how these activities are coupled with those in the aerobic layers and water columns provide especially difficult, but challenging, problems. The role of the sea in global cycles of carbon, nitrogen and sulfur need further study. For example, the sea has not proved to be as efficient a "sink" for CO_2 released into the atmosphere by fuel-burning and deforestation, as had once been predicted. Finally, the impact of estuaries and coastal wetlands on continental shelf waters needs close reexamination on local and regional scales. We now have a pretty good understanding of upwelling processes, but not of outwelling processes. On the basis of our early work at Sapelo, we thought that the salt marsh estuaries exported large quantities of detritus, but now we are not so sure if it's POC, DOC, or living biomass that outwells, if indeed there is a net export at all. There is likely wide regional variation in import and export flows of carbon and nutrients along our coastline, and these need to be quantified if we are to anticipate the fate of pollutants which in the future are going to be introduced offshore (offshore drilling, etc.) as well as inshore.

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PREPARATION AND CHARACTERIZATION OF A MARINE REFERENCE MATERIAL FOR TRACE ELEMENT DETERMINATIONS

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ABSTRACT

A reference material for marine molluscan trace element determinations has been developed. It consists of 637 clams, *Arctica islandica,* that have been homogenized together and subsequently divided into 476 samples. A representative subsample of these has been analyzed for trace element concentrations. Of the 14 elements measured, 10 had relative standard deviations from the mean of 7% or less.

INTRODUCTION

The study of pollution in marine systems often involves the measurement of trace element concentrations in organisms over extended periods of time (1, 2). Development of valid time trends from such data requires a strict quality control program at every stage of data collection, from field sampling through final statistics, to ensure that data from any single point in time is comparable to that collected at all other times. This paper describes some of the efforts undertaken to provide control over the laboratory analysis of marine organisms for trace element concentrations. Specifically, we describe the preparation and characterization of an in-house reference material which can be used as a benchmark sample for quality control, a known sample for methods development, or an intercalibration sample.

EXPERIMENTAL

Marine molluscan samples are prepared for flame atomic absorption spectroscopy as follows (3):

1. Thaw sample.
2. Using stainless steel instrument, shuck into a dry, labeled, tared beaker. Determine wet weight.

3. Cover beaker with watch glass and place in drying oven. Dry to constant weight at 95°C (about 48 hours).

4. Cool and determine dry weight.

5. Add concentrated nitric acid in sufficient quantity to cover organisms, adding acid in 20 ml increments. Cover beaker with watch glass. (Use same amount of acid for all samples within a group.)

6. Let sample cold digest until tissue is well broken down; i.e., 4-24 hours.

7. Heat gently, to about 40°C, being careful to avoid frothing over. Continue until frothing stops.

8. Heat to 85°C while covered, and bring sample to near dryness. (*Be careful not to take to complete dryness at any time.*)

9. Remove from hot plate and add 20 ml of concentrated nitric acid. Repeat step 8.

10. Repeat step 9 until digestion is complete, which is indicated by pale yellow color, clarification of the liquid, and no trace of lipids. (Treat all samples within a group in identical fashion.)

11. Take to near dryness (about 5 ml remaining), cool, and add 20 ml of 5% nitric acid, getting all soluble residue into solution.

12. Filter sample into 50 ml volumetric flask through Whatman #42 Filter Paper which has been prerinsed with 5% nitric acid.

13. Rinse beaker 2-3 times with 5% nitric acid and pour through filter.

14. Rinse funnel down and bring up to volume with 5% nitric acid.

15. Mix well, transfer to acid stripped 60 ml polyethylene bottle, and hold for A.A. analysis.

NOTE: All glassware is detergent washed, soaked in 10% nitric acid, and copiously rinsed with deionized water.

Each group of 15 samples is accompanied by a complete reagent methods blank. Analysis is performed on a Perkin-Elmer model 603 Atomic Absorption spectrometer according to the manufacturer's instructions. Operation is facilitated by the use of an autosampler (P.E. Auto 200) and an ASR-33 teletypewriter. To ensure against instrument drift, a calibration standard is included with each 15 samples. To check for unknown matrix effects, a known spike is added to an aliquot of one of the samples and an equal quantity of 5% nitric acid. From this, a spike recovery is calculated for each group of 15. For each group, a sample of the reference material described in this paper is included.

Preparation of Reference Material

Clams (*Arctica islandica*) were collected by commercial dredge from Block Island Sound, and frozen prior to use. At the time of preparation, they were cleaned, thawed and shucked as if for analysis, except that extracellular fluid was drained and discarded. A total of 637 clam meats was pureed and homogenized in a stainless steel, 40 quart mixer, of the sort found in many commercial kitchens (Hobart VCM-40). Samples of approximately 60 g wet weight were removed, placed in 120 ml acid-stripped polyethylene bottles, sequentially numbered, and frozen for future analysis. This procedure yielded 476 samples.

Characterization of Reference Material

From the 476 samples, a total of 65 were selected for investigation of the homogeneity of the material. These consisted of every tenth sample, and two blocks of 10 consecutive samples from each end of the sequence. These samples were analyzed for 14 trace elements by the above procedure, and the results examined for homogeneity.

RESULTS AND DISCUSSION

The concentrations of 14 metals in the reference material on a wet weight basis are listed in Table 1-1. Only data on a wet weight basis will be discussed because of some anomalous wet to dry weight ratios indicating some samples were not uniformly dried. On the basis of several criteria, the 14 metals may be divided into two groups, A and B. Group A consists of the 10 metals Cd, Cr, Cu, Fe, Mg, Mn, Ni, Pb, V, and Zn, for which the relative standard deviations are less than 7%, as seen in Table 1-2. Three of these metals are graphically represented in Figure 1-1. Note the similarity of the graphs. This similarity may be quantified for these metals by determining the 45 pair-wise correlation coefficients. Almost all of the coefficients indicate positive correlation at the 95% confidence level, with many of them being much more highly significant.

Table 1-1. Trace Element Concentrations in Reference Material, ug/g (Wet)

	GROUP A										GROUP B			
Sample Number	Cd	Cr*	Cu	Fe	Mg	Mn	Ni	Pb	V	Zn	Al	Ca	Co	Ti
10	0.18	1.02	2.87	92.3	816	3.25	1.57	1.27	0.9	17.5	19.3	422	0.22	1.0
20	0.18	0.94	2.82	90.8	1040	3.19	1.45	1.29	0.9	17.7	16.7	541	0.19	0.9
30	0.18	1.00	2.90	86.4	857	3.25	1.48	1.30	0.9	17.6	16.4	499	0.27	0.9
40	0.17	0.98	2.85	92.3	776	3.19	1.49	1.31	0.9	17.5	18.5	419	0.16	1.0
50	0.18	0.96	2.85	91.1	760	3.19	1.55	1.32	0.9	17.7	18.2	387	0.21	1.0
60	0.18	0.98	2.85	90.3	830	3.28	1.47	1.28	0.9	17.8	15.9	437	0.27	0.9
61	0.17	1.00	2.84	88.3	855	3.14	1.51	1.26	0.9	17.3	17.9	453	0.23	0.7
62	0.18	1.01	2.83	88.2	870	3.18	1.51	1.26	0.9	17.6	19.0	462	0.12	1.1
63	0.17	1.00	2.84	87.0	757	3.07	1.51	1.25	0.9	17.0	16.9	389	0.15	0.9
64	0.18	0.98	2.84	87.6	888	3.16	1.54	1.32	0.9	17.6	16.7	459	0.15	0.6
65	0.18	1.06	2.87	95.4	776	3.25	1.53	1.33	0.9	16.8	27.5	396	0.25	0.9
66	0.17	0.99	2.81	91.3	790	3.15	1.54	1.30	0.9	17.4	18.3	411	0.23	1.0
67	0.17	0.94	2.75	88.1	801	3.02	1.43	1.25	0.8	17.2	16.6	461	0.19	0.6
68	0.18	1.02	2.82	91.5	840	3.21	1.48	1.30	0.9	17.9	18.9	563	0.21	0.9
69	0.18	1.05	2.82	85.9	767	3.06	1.44	1.26	0.9	17.4	15.6	489	0.11	0.8
70	0.17	1.12	2.82	95.6	792	3.10	1.54	1.25	0.9	17.3	17.2	479	0.19	1.1
80	0.18	1.02	2.83	95.4	742	3.22	1.49	1.26	0.9	17.8	20.0	369	0.24	1.1
90	0.17	1.05	2.87	92.6	775	3.20	1.47	1.28	0.9	17.9	20.1	368	0.23	1.2
100	0.17	1.02	2.79	92.2	731	3.17	1.46	1.26	0.8	17.5	18.6	403	0.23	1.0
110	0.18	1.03	2.79	90.4	765	3.10	1.51	1.24	0.9	17.5	20.0	369	0.20	0.6
120	0.17	1.00	2.75	85.5	765	3.05	1.50	1.24	0.8	17.6	19.1	400	0.21	0.7
130	LOST													
140	0.16	1.04	2.86	90.0	813	3.14	1.49	1.24	0.8	17.4	18.8	432	0.23	1.0
150	0.17	1.02	2.68	88.7	865	3.14	1.42	1.13	0.8	17.3	19.8	589	0.22	0.8
160	0.18	1.05	2.82	91.1	803	3.26	1.52	1.27	0.9	17.4	21.7	409	0.18	0.6
170	0.18	1.01	2.84	94.6	839	3.18	1.52	1.24	0.9	17.3	20.2	414	0.18	1.3
180	0.16	1.00	2.74	94.5	849	3.09	1.38	1.19	0.9	17.6	21.3	481	0.21	0.3
190	0.17	1.08	2.84	92.0	781	3.27	1.50	1.26	0.9	17.3	22.1	430	0.25	<0.3
200	0.17	1.03	2.86	90.0	808	3.11	1.49	1.25	0.9	17.6	20.2	439	0.19	1.1
210	0.18	1.08	2.84	96.4	894	3.16	1.49	1.30	1.0	17.5	21.3	464	0.17	1.4
220	0.18	1.05	2.85	87.5	824	3.12	1.47	1.29	0.9	17.2	20.4	412	0.20	0.9
230	0.18	1.02	2.76	93.2	680	3.01	1.51	1.20	0.9	17.6	20.1	337	0.11	0.4
240	0.18	1.01	2.81	87.4	781	3.18	1.46	1.30	0.8	17.2	18.0	389	0.15	1.0
250	LOST													

*Corrected for blank

Table 1-1. (Continued)

	GROUP A										GROUP B			
Sample Number	Cd	CR*	Cu	Fe	Mg	Mn	Ni	Pb	V	Zn	Al	Ca	Co	Ti
260	0.17	1.03	2.84	85.3	767	3.17	1.47	1.29	0.9	17.6	19.5	444	0.20	1.1
270	0.17	1.06	2.83	86.0	730	3.07	1.56	1.28	0.9	17.3	18.6	375	0.21	0.9
280	0.16	0.99	2.82	92.2	790	3.18	1.49	1.31	0.9	17.6	17.5	424	0.18	1.1
290	0.16	1.03	2.81	89.3	782	3.10	1.22	1.27	0.9	17.5	18.4	446	0.18	1.1
300	0.17	1.00	2.82	89.0	777	3.08	1.38	1.31	0.9	17.2	16.9	417	0.22	0.9
310	0.17	1.04	2.83	91.9	748	3.22	1.49	1.28	0.9	16.9	18.0	401	0.19	1.1
320	0.17	1.00	2.78	88.0	758	3.12	1.50	1.27	0.9	16.8	15.9	381	0.19	0.8
330	LOST													
340	0.17	1.02	2.78	89.4	793	3.03	1.45	1.29	0.8	17.0	14.9	454	0.24	0.6
350	0.20	1.08	3.13	99.2	898	3.43	1.67	1.42	0.9	19.0	19.1	452	0.21	1.0
360	0.16	0.94	2.56	75.4	649	2.80	1.40	1.18	0.8	15.3	14.8	327	0.19	0.8
370	0.18	1.04	2.83	79.4	782	3.22	1.47	1.21	0.8	16.7	12.7	456	0.15	0.6
380	0.19	1.05	2.87	83.8	870	3.10	1.45	1.24	0.8	17.1	15.4	463	0.15	1.0
390	0.18	0.94	2.85	87.0	806	3.13	1.54	1.28	0.9	17.4	18.1	462	0.13	0.6
400	0.19	0.96	2.82	87.0	747	3.13	1.50	1.24	0.9	17.1	14.6	496	0.22	0.5
401	0.18	1.00	2.84	84.9	775	3.10	1.52	1.27	0.9	16.9	14.6	369	0.19	0.6
402	0.18	1.04	2.86	87.4	821	3.15	1.49	1.33	0.9	17.2	16.5	420	0.18	1.0
403	0.18	1.12	2.84	78.5	823	3.29	1.45	1.30	0.8	17.0	14.7	734	0.23	0.5
404	0.18	1.00	2.80	80.2	787	3.07	1.44	1.29	0.9	16.9	12.8	406	0.13	0.6
405	0.17	0.88	2.80	88.6	775	3.10	1.48	1.30	0.8	17.3	16.1	427	0.14	0.5
406	0.17	0.90	2.82	90.7	759	3.21	1.47	1.30	0.8	16.7	15.8	394	0.18	0.7
407	0.17	1.00	2.80	87.2	803	3.15	1.45	1.26	0.9	17.6	17.4	421	0.20	0.8
408	0.18	1.04	2.90	90.0	774	3.13	1.44	1.30	0.9	17.6	19.1	437	0.20	0.9
409	0.17	1.01	2.82	85.6	810	3.11	1.41	1.32	0.9	16.9	15.4	470	0.19	0.5
410	0.18	1.03	2.80	84.6	885	3.14	1.48	1.29	0.9	17.9	18.8	453	0.16	0.8
420	0.17	1.03	2.82	88.4	776	3.15	1.45	1.29	0.9	17.4	17.0	422	0.18	1.0
430	0.17	1.02	2.85	91.1	854	3.20	1.40	1.27	0.9	17.3	17.0	452	0.21	1.0
440	0.15	0.93	2.59	77.4	781	2.87	1.36	1.17	0.8	15.7	14.6	456	0.21	0.7
450	0.17	1.00	2.75	87.3	869	3.10	1.43	1.26	0.9	17.2	15.7	468	0.20	0.9
460	0.19	1.07	2.76	89.1	823	3.45	1.56	1.33	1.0	17.9	18.2	492	0.23	0.8
470	0.14	0.99	2.82	83.7	782	3.23	1.45	1.32	0.9	16.9	15.9	439	0.21	<0.4

*Corrected for blank

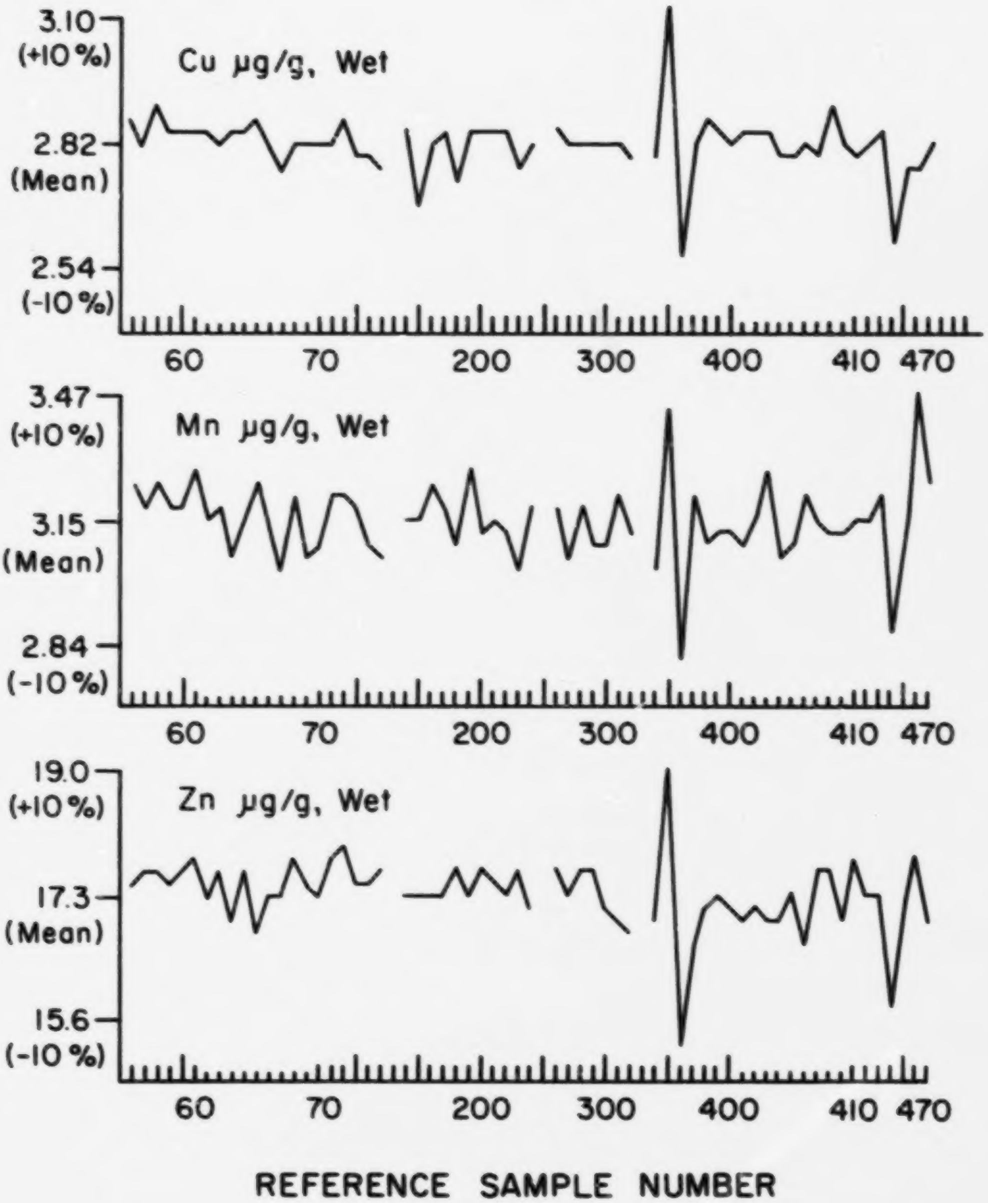

Figure 1-1. Trace element distributions in reference material.

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Table 1-2. Relative Standard Deviations

Metal	Mean, ug/g Wet	St. Dev.	Rel. Std. Dev.
Group A			
Cd	0.17	0.01	6
Cr	1.01	0.05	5
Cu	2.82	0.07	3
Fe	88.7	4.5	5
Mg	802	58	7
Mn	3.15	0.10	3
Ni	1.48	0.06	4
Pb	1.27	0.04	3
V	0.9	(0.05)	6
Zn	17.3	0.5	3
Group B			
Al	17.8	2.5	14
Ca	439	61	14
Co	0.19	.04	21
Ti	0.8	0.3	38

In contrast are the group B metals which consist of Al, Ca, Co, and Ti. As shown in Table 1-2, these metals are characterized by a much higher relative standard deviation, from 14% to 38%. When plotted in a fashion similar to Figure 1-1, they show a much higher degree of scatter. Accordingly, the correlation coefficients show no real correlations at the 95% confidence level.

Although this material has been prepared as a reference material, it is in no way equivalent to a standard reference material such as those developed by the National Bureau of Standards (NBS) (4). The NBS Standard Reference Materials are certified as to their trace element content on the basis of analysis by at least three independent techniques. Our material is an internal reference material, not a certified standard.

This material is now in routine use within our laboratory. One sample, randomly chosen from the sequence, is included with each group of 15 unknown samples prepared and analyzed. Thus, there is continuous verification of analytical results irrespective of operator, instrument settings, or standards. In addition, this material is ideal for methods development and intercalibration because of its extremely low variability. Individual organisms from a wild population can have a range of metal concentrations up to an order of magnitude, thus obscuring differences between techniques that may be small but real. Comparison of clam homogenate values, however, will render obvious differences of less than 25% which would be arduous or impossible to discern using natural populations.

CONCLUSIONS

We have shown that a satisfactorily homogeneous reference material can be prepared with a minimum of specialized equipment. The material has a low relative standard deviation for the 10 metals Cd, Cr, Cu, Fe, Mg, Mn, Ni, Pb, V, and Zn. For reasons that are not always clear, the other metals analyzed Al, Ca, Co, and Ti have higher relative standard deviations. The material is useful as a benchmark reference material so that analyses at different times can be shown to be intercomparable. Thus, legal defensibility of time series studies of pollution sites can be greatly enhanced. The material is also ideal for methods development and intercalibrations because of its very low relative standard deviation. Therefore, with cooperation from a cafeteria kitchen, it becomes a relatively simple task to prepare such a reference material when a certified standard from a recognized supplier such as NBS is not available.

ACKNOWLEDGEMENTS

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THE RELEASE OF HEAVY METALS FROM REDUCING MARINE SEDIMENTS

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ABSTRACT

We address the hypothesis that metals forming soluble sulfides are released from nearshore anoxic sediments, while those forming insoluble sulfides are retained. As a test, we have studied pore water chemistry, benthic fluxes, and water column distributions of heavy metals in Narragansett Bay, Rhode Island. The results show that metal forming soluble sulfides (Mn and Fe) have high pore water concentrations and are released to the Bay waters, while metals forming insoluble sulfides have low pore water concentrations and negligible benthic fluxes.

INTRODUCTION

Several authors have suggested that heavy metal concentrations in reducing marine waters are strongly influenced by sulfide solubility: metals forming relatively soluble sulfides (such as Fe and Mn) will have relatively high concentrations, and those forming relatively insoluble sulfides (such as Cd and Cu) will have very low concentrations (5, 13, 4). A corollary of this suggestion is that benthic fluxes (fluxes of dissolved chemicals from sediments to the overlying water) out of anoxic sediments should be high for those metals forming soluble sulfides, and negligible or even negative for those metals forming insoluble sulfides. These hypotheses, if correct, have important implications. They imply that estuarine and reducing nearshore and continental shelf sediments are not sites where insoluble sulfide-forming metals are readily remobilized: metals reaching these sites by inorganic scavenging or biological removal are, most likely, permanently sequestered as long as sulfide is being produced in the sediment porewaters. Secondly, the hypotheses indicate that, to avoid release of metals forming insoluble sulfides, sewage sludge and dredge spoils polluted with heavy metals should be dumped under sites of high organic productivity, where continued deposition of organic matter maintains the conditions which prevent their release to the overlying water. Of course, other considerations (such as the presence of organic toxins) may show another course of action to be more prudent.

We have carried out a series of experiments aimed in large part at testing the two hypotheses outlined above. We have proceeded by 1) determining the concentrations of elements forming relatively soluble sulfides (Mn and Fe) and elements forming relatively insoluble sulfides (Ni, Cu and Cd), along with nutrients, pH, total CO_2 (TCO_2), $SO_4^=$, and $S^=$ in anoxic Narragansett Bay sediments; 2) carrying out tracer experiments to determine the rate at which organisms enhance the benthic flux of chemicals by pumping water across the sediment-water interface, and using these results, along with diffusive flux estimates, to predict total fluxes across the sediment-water interface; 3) measuring benthic fluxes directly via bell jar experiments, and using the results both to check the prediction of the pore water data and flux model, and to make direct determinations of metal benthic fluxes; and 4) working out the mass balance of heavy metals in Narragansett Bay at one point in time, using the Bay itself as a gigantic bell jar and thereby checking on the generality of our benthic flux measurements.

In this paper we summarize the results of this work.

PORE WATER WORK

Samples were collected in plexiglas box cores or PVC pipe. One centimeter sediment slices were placed in polyethylene centrifuge bottles in a helium atmosphere and centrifuged at *in situ* temperatures to separate pore waters. Supernatant waters were filtered through acid-washed Nuclepore filters. The concentration of TCO_2 was determined by gas chromatography. Sulfate was measured by $BaSo_4$ coprecipitation using ^{133}Ba as a tracer. Sulfide and ammonia were determined colorimetrically by the methods of Cline (3) and Solorzano (10), respectively. Phosphate and silicate were determined by a modified autoanalyzer procedure (11, 12). Dissolved manganese and iron were determined by flameless atomic absorption spectrometry using either a Perkin-Elmer 503 or 360 AAS with model 2100 graphite furnace deuterium arc background corrector. Standards were prepared in Sargasso Sea water. Further details of sample collection and the above analytical methods are given by McCaffrey (8).

Cd and Ni were determined by flameless atomic absorption after sample concentration using a modification of the Co-APDC coprecipitation technique of Boyle and Edmond (2). A 0.2 ml aliquot of a 4m$\underline{M}$ $CoCl_2$ solution is added to the pore water sample at pH 2, followed by addition of 0.4 ml of a 2% W/V solution of APDC. The sample is shaken vigorously and allowed to sit for up to 30 minutes. It is then filtered through a 2.5 cm diameter, 0.4 μm micron Nuclepore filter held by an acid-washed Millipore filter apparatus. The filter is washed with several ml of deionized water, and placed in a polypropylene vial containing 1.0 ml of redistilled 3N HNO_3. The precipitate is dissolved by ultrasonication for 1 hour. The filter is then removed and the solution analyzed by flameless atomic absorption.

The yields, as determined by addition for Ni and Cu and tracer experiments for Cd, are 0.76±.06%, 0.75±.10, 0.70±.08 for Ni, Cu and Cd, respectively. The precision is about ±10% at concentrations well above the detection limits of 0.1 ppb for Cd and 1.0 ppb for Cu and Ni.

Equipment and reagents are all carefully cleaned prior to Ni, Cu and Cd analysis. Polypropylene vials are soaked in 6$\underline{M}$ HCl for 18 hours, rinsed with deionized water, and ultrasonicated twice for three hours each time in 6$\underline{M}$ HCl. They are rinsed at least three times and dried. $CoCl_2$ $6H_2O$ is purified using Dowex-1-X8 anion exchange resin. It is put on in 9$\underline{M}$ HCl and eluted in 4$\underline{M}$ HCl. The APDC solution is purified by filtration through a 0.4 μm micron Nuclepore filter followed by five extractions with 20 ml MIBK (methyl isobutyl ketone). Filters are acid washed, as all glassware used in the filtration is continuously soaked in acid. Blanks are below the detection limits.

Typical summer pore water profiles are shown in Figures 2-1 and 2-2 for a long core and four short cores from the Jamestown North study site in Narragansett Bay (located about 0.5 km north of Jamestown Island in 5-10 m of water). These profiles are discussed in detail by McCaffrey et al (8). Concentrations of all constituents are far higher in the top centimeter than in bottom water (TCO_2 increases from 2.0 to 2.8 m$\underline{M}$, NH_3 from ~5 to 100 $\mu\underline{M}$, $PO_4^{=}$ from 1-25 $\mu\underline{M}$, etc.). From 1-20 cm the profiles are flat (TCO_2, NH_3, H_4SiO_4) or show decreasing concentrations with depth ($PO_4^{\equiv}$, Mn^{++}). The lack of a systematic increase is ascribed to transport of metabolites out of the sediments by the pumping activity of organisms, rather than by ionic or molecular diffusion. The sharp concentration decreases observed for Mn^{++} and $PO_4^{\equiv}$ are ascribed to inorganic reaction in the sediment column. Below approximately 25 cm, $SO_4^{=}$ concentrations decrease and concentrations of other metabolites increase. Organisms are assumed to be absent, or at least ineffective water transporters, below this depth. Metabolite concentrations change sympathetically approximately as predicted by organic matter decomposition: TCO_2 increases twice as fast as $SO_4^{=}$ decreases NH_3 increases about 1/7 as rapidly as TCO_2, and $PO_4^{\equiv}$ increases about 1/150 as rapidly as TCO_2.

An important point about the flat portion of the profiles is that the TCO_2 value is considerably higher than can be accounted for by O_2 reduction, and implies anoxic diagenesis. The bottom water TCO_2 and O_2 concentrations are 2.0 and 0.15 m$\underline{M}$, respectively. When all O_2 is consumed, the TCO_2 concentration will rise to 2.15 m$\underline{M}$. NO_3 reduction could conceivably increase the value to 2.2 m$\underline{M}$. $SO_4^{=}$ reduction must be postulated as the agent causing the further increase to 2.8 m$\underline{M}$.

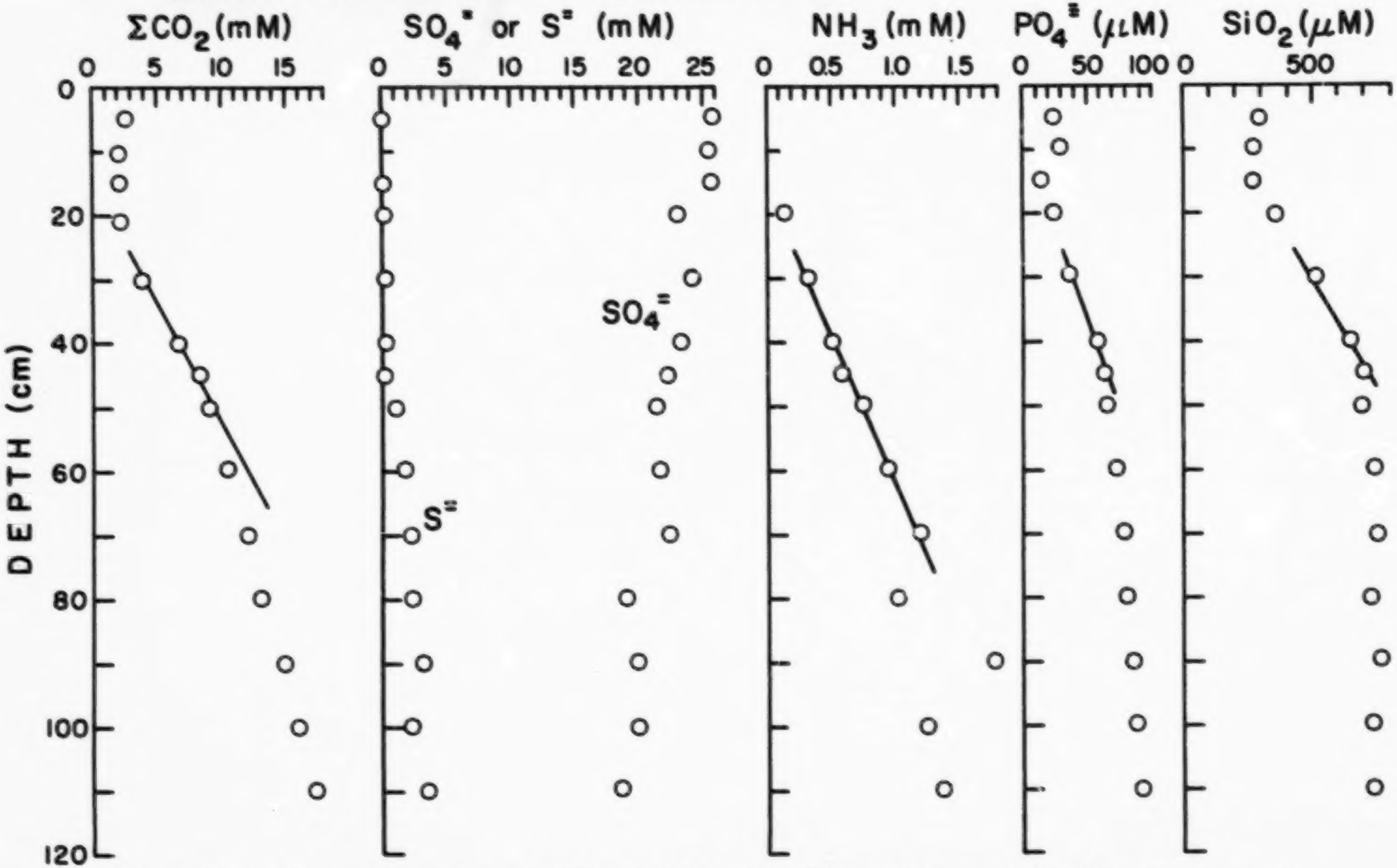

Figure 2-1. Concentrations of TCO_2, $SO_4^=$, $S^=$, NH_3, $PO_4^=$, and $H4SiO_4$ in pore waters from a Jamestown North long core (JN-8) (from McCaffrey et al, 1977).

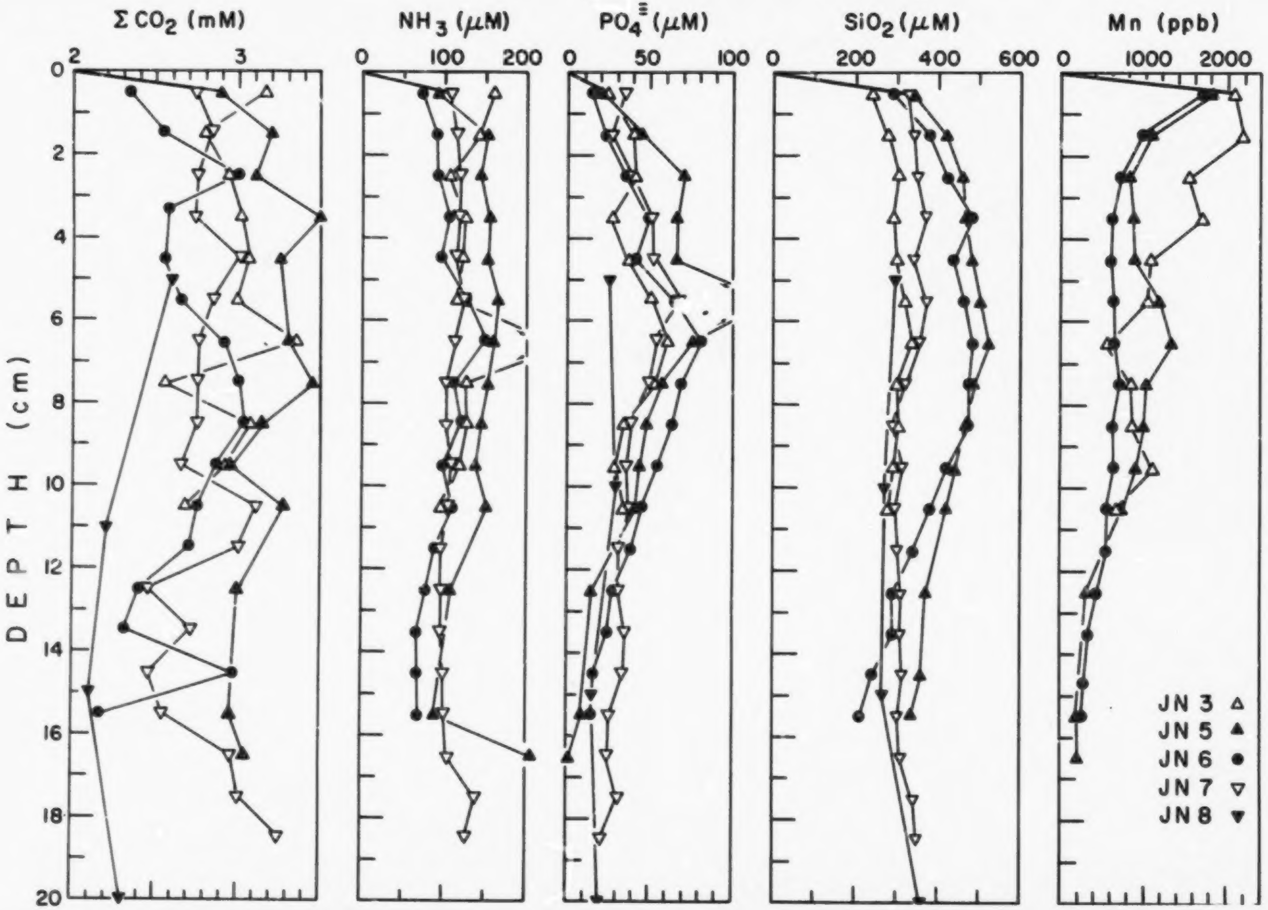

Figure 2-2. Concentrations of TCO_2, NH_3, $PO_4^{\equiv}$, H_4SiO_4 and Mn^{++} vs. depth in pore waters of the long core (NJ-8) and four short cores from the Jamestown North study site.

Note: Heavy lines show inferred concentration gradients at the sediment-water interface (from McCaffrey et al, 1977).

Nutrient and metal data for Jamestown North core 11 (a short core collected on 7/13/76) and 12 (a long core collected on the same date) are given in Tables 2-1 and 2-2. Metabolite concentrations vary in the manner discussed earlier.

The metal concentrations are striking. Mn and Fe values are similar to values reported elsewhere for anoxic sediments (11). Cu and Cd concentrations, on the other hand, are very low, being comparable to or less than Bay bottom values of about 2 ppb (Cu) and 0.2 ppb (Cd) at the Jamestown North location. There is some scatter in the Cu and Cd data. This is in part due to contamination. For example, the 58-60 cm sample in Jn-12 has Mn, Fe and Cd values which are all higher than values in surrounding samples, apparently due to contamination. On the other hand, there may be real variations, such as a Cu maximum at 3-6 cm in JN11. Nevertheless, the main conclusion is clear: of the four metals we analyzed, those forming relatively soluble sulfide (Mn and Fe) gave pore water concentration far above ambient bottom waters, whereas those forming highly insoluble sulfides (Cu and Cd) gave concentrations comparable to or less than bottom water values. We would not expect such sediments to release Cu and Cd to the bottom waters at significant rates.

MODELLING SEDIMENT-WATER EXCHANGE

To understand how fluxes of constituents between pore and overlying waters at Jamestown North depend on pore water and bottom water distributions, McCaffrey et al (8) constructed a simple model in which we consider both exchange mechanisms – simple diffusion (transport along gradients due to the thermal motion of ions and molecules) and advection (transport in water which is moving as a result of the irrigation, feeding and burrowing activities of the benthic fauna).

Conceptually, the diffusive flux is easy to calculate – it is simply the product of a diffusion coefficient and a concentration gradient. Diffusion coefficients in Narragansett Bay sediments at 25°C were taken as half the value at 25°C in deionized water. Concentration gradients are not well known: the gradients are very steep in the top 1 centimeter and zero below, and the pore water concentrations do not allow us to accurately estimate the gradients at the interface. We have assumed that the concentration near the interface is as shown in Figure 2-2. Diffusive fluxes were calculated from the product of the gradient and the diffusion coefficient.

Aller (1) and others have stressed the importance of organisms in sediment-water exchange. In calculating advective fluxes, it is necessary to know the rate at which organisms move water. The activity of organisms may either be modelled as a random, or "biodiffusion" process, or as an ordered, or "biopumping" process. Since organisms pump water into the sediment to

Table 2-1. Nutrient and Metal Concentrations in Pore Waters from a Short Core from the Jamestown North Study Site (JN-11)

Depth (cm)	TCO_2 (m$\underline{M}$)	$SO_4^=$ (m$\underline{M}$)	$S^=$ (m$\underline{M}$)	NH_3 ($\mu\underline{M}$)	$PO_4^=$ ($\mu\underline{M}$)	Mn (ppb)	Fe (ppb)	Cd (ppb)	Cu (ppb)	Ni (ppb)
0–1/2	2.83	26.2	<0.003	75	13.0	2500	2000	<.10	2.6	5.8
1/2–1	2.73	25.7	–	82	31	1580	5700	<.10	<1	3.4
1–2	2.89	–	<0.003	101	50	890	5700	<.10	1.6	3.1
2–3	2.98	25.8	–	104	61	700	5500	<.10	1.8	1.6
3–4	2.75	25.2	<0.003	90	35	290	3200	.19	7.0	2.2
4–5	2.56	26.0	<0.003	88	35	230	3100	<.10	3.2	1.8
5–6	2.58	24.7	<0.003	82	27	240	2400	<.10	2.6	1.8
6–7	2.71	25.8	<0.003	71	22	240	1800	<.10	2.2	2.0
7–8	2.94	25.1	<0.003	72	22	230	1440	<.10	1.8	3.0
8–9	2.52	26.0	<0.003	68	18.8	195	800	<.10	<1	1.6
9–10	2.47	25.4	<0.003	62	13.0	220	250	<.10	<1	1.9
10–11	2.40	26.2	<0.003	58	15.0	170	–	<.10	<1	2.3
11–12	2.75	26.3	<0.003	57	12.1	160	85	.16	1.4	4.1
12–13	2.35	26.5	<0.003	51	11.1	155	42	.16	<1	2.6
13–14	2.50	26.2	<0.003	45	10.1	148	44	<.10	8.4	13.4
14–15	2.50	24.3	0.003	41	8.7	122	101	.10	<1	2.6

Table 2-2. Nutrient and Metal Concentrations in Pore Waters from a Long Core from the Jamestown North Study Site (JN-12)

Depth (cm)	TCO_2 (m$\underline{M}$)	$SO_4^=$ (m$\underline{M}$)	$S^=$ (m$\underline{M}$)	NH_3 ($\mu\underline{M}$)	$PO_4^=$ ($\mu\underline{M}$)	Mn (ppb)	Fe (ppb)	Cd (ppb)	Cu (ppb)
4–6	3.17	26.0	<0.003	120	15.5	210	155	< .10	<1
10–12	2.58	25.5	<0.003	64	10.1	135	50	.34	<1
16–18	2.76	25.3	<0.003	97	9.7	114	80	< .10	<1
22–24	3.11	24.9	0.011	161	20	83	60	< .10	<1
28–30	4.23	24.4	0.036	250	29	28	45	< .10	<1
34–36	5.41	24.8	0.030	240	41	11	42	< .10	<1
40–42	6.33	25.8	0.102	410	47	9	62	< .10	<1
46–48	7.16	23.3	0.39	480	58	11	67	< .10	<1
52–54	8.29	21.6	0.32	590	65	26	56	< .10	<1
58–60	9.12	20.1	0.38	640	75	32	101	2.29	<1
67–70	10.4	22.8	0.51	740	80	8	47	< .10	<1
77–80	11.0	20.7	0.74	810	85	6	58	< .10	<1
87–90	12.3	22.5	0.60	860	94	1	42	< .10	<1
96–99	12.4	21.2	0.67	950	92	10	55	< .10	<1

supply oxygen and pump water out to expel waste products, we regarded the biopumping model as more appropriate. In the model of McCaffrey at al (8), it was assumed that organisms pumped water across the sediment-water interface at a certain "biopumping rate" (with units of volume of water per unit surface area per unit time). The biopumping rate was determined experimentally by bringing box cores of Jamestown North sediment into the laboratory, spiking the supernatant solution with ^{22}Na, and measuring the decrease in the supernatant ^{22}Na concentration with time. The experiments gave a biopumping rate of 0.7±0.3 cm^3 cm^{-2} day^{-1}. The biopumping flux is then taken as the product of the biopumping rate and the difference between pore water and bottom water concentrations.

Model diffusive and advective fluxes for summer Jamestown North sediments are given in Table 2-3. Surprisingly, both fluxes are of the same magnitude.

RESULTS OF BENTHIC FLUX MEASUREMENTS

In order to test our predictions that nutrient and manganese fluxes have the values calculated from the model outlined in the previous section, and to make direct measurements of copper and nickel fluxes, we have measured benthic fluxes in the field using the "bell jar" instruments developed and extensively deployed by Hale (7) and Nixon et al (9). In these experiments, PVC pipe halves with closed ends PVC flanges around the base are placed on the sediment. At the start of the experiment, a sample is withdrawn from the

Table 2-3.

	Diffusive Flux	Advective flux calculated from biopumping model*	Measured fluxes: $\bar{x} \pm 1\sigma(n)$
H_4SiO_4	0.3	0.3	1.2±0.2(7)
NH_3	0.19	0.07	0.27±0.08(15)
$PO_4^{\equiv}$	0.018	0.02	0.07±0.02(9)
ΣCO_2	0.9	0.6	2(1)
Mn^{++}	0.02	0.01	0.049±0.018(14)

*Assuming a biopumping rate of 0.7 cm^3 cm^{-2} day^{-1}

chamber from a valve at one end; a second sample is withdrawn at the end of the experiment. An ambient bottom water sample is also taken, and a dark bottle is filled with water at the start of the experiment and sampled at the end. The samples are analyzed for metals and nutrients after filtration. Benthic fluxes are calculated from the change in the concentration of a constituent in a chamber, the mean height of the chamber (chamber volume/enclosed sediment surface area), and the length of the experiment. Dark bottle "fluxes" are calculated in the same manner as benthic fluxes, taking the dark bottle concentration at the end of the experiment as the final concentration, and the ambient bottom water concentration as the initial concentration. The dark bottle results are important for metals; because if they are equal to zero within analytical uncertainties, they indicate that metal analyses are not seriously affected by sample contamination during collection.

Metal concentrations were measured with the techniques used for pore waters. The ambient water column metal concentrations at Jamestown North are about 10 ppb (0.2 $\mu \underline{M}$) for Mn and Fe, 3 ppb (0.06 $\mu \underline{M}$) for Ni, 2 ppb (0.04 $\mu \underline{M}$) for Cu, and 0.1 ppb (.001 $\mu \underline{M}$) for Cd. Precisions in individual metal analyses are ±10%; hence uncertainties in fluxes are 40 $\mu M\ m^{-2}\ day^{-1}$ for Mn and Fe, 8 $\mu M\ m^{-2}\ day^{-1}$ for Cu and Ni, and 2 $\mu M\ m^{-2}\ day^{-1}$ for Cd. Histograms of dark bottle results at Jamestown (Figure 2-3) are roughly as predicted from these errors.

Results of experimentally determined fluxes of Mn^{++} and nutrients at Jamestown North are given in Table 2-3, along with model fluxes for these chemicals presented earlier. The model fluxes agree quite well with the measured values, indicating that the system is well characterized.

A histogram of Jamestown North metal fluxes is shown in Figure 2-4 , and averages are tabulated in Table 2-4. Manganese fluxes are similar to the model values. Pore water iron concentrations are similar to those of manganese, and comparable fluxes are predicted. Observed iron fluxes are about an order of magnitude lower than manganese (and predicted) fluxes; this is ascribed to rapid oxidation of iron in the supernate following diffusion out of sediments. Nickel, copper, and cadmium fluxes are predicted to be negligible (see discussion of pore water values, above) and in fact measured fluxes are generally equal to zero within the analytical uncertainty.

From the concentration of constituents in the Bay, the average height of the water column (taken as 10 m) and the fluxes, we can calculate doubling times of metals in the Bay with respect to benthic fluxes. These values are given in Table 2-4. Upper limits on Cu, Ni and Cd doubling times were calculated taking the flux as less than or equal to the sum of the mean flux and one standard deviation. Doubling times are to be compared with residence times of water in Narragansett Bay of about one month. The results show that, in the

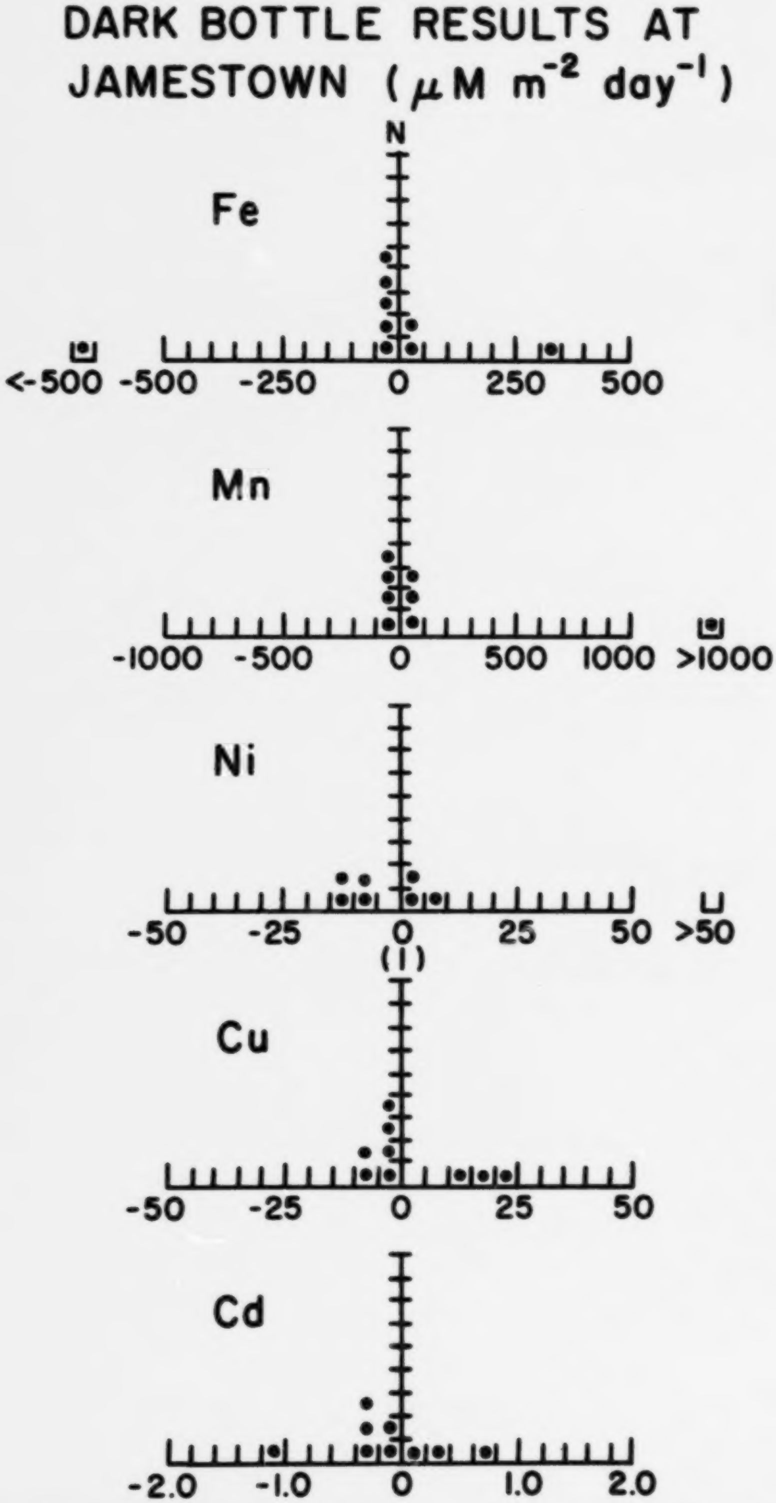

Figure 2-3. Histograms of dark bottle results at Jamestown North, calculated as fluxes (see text). Units are $\mu\underline{M}$ m^{-2} day^{-1}.

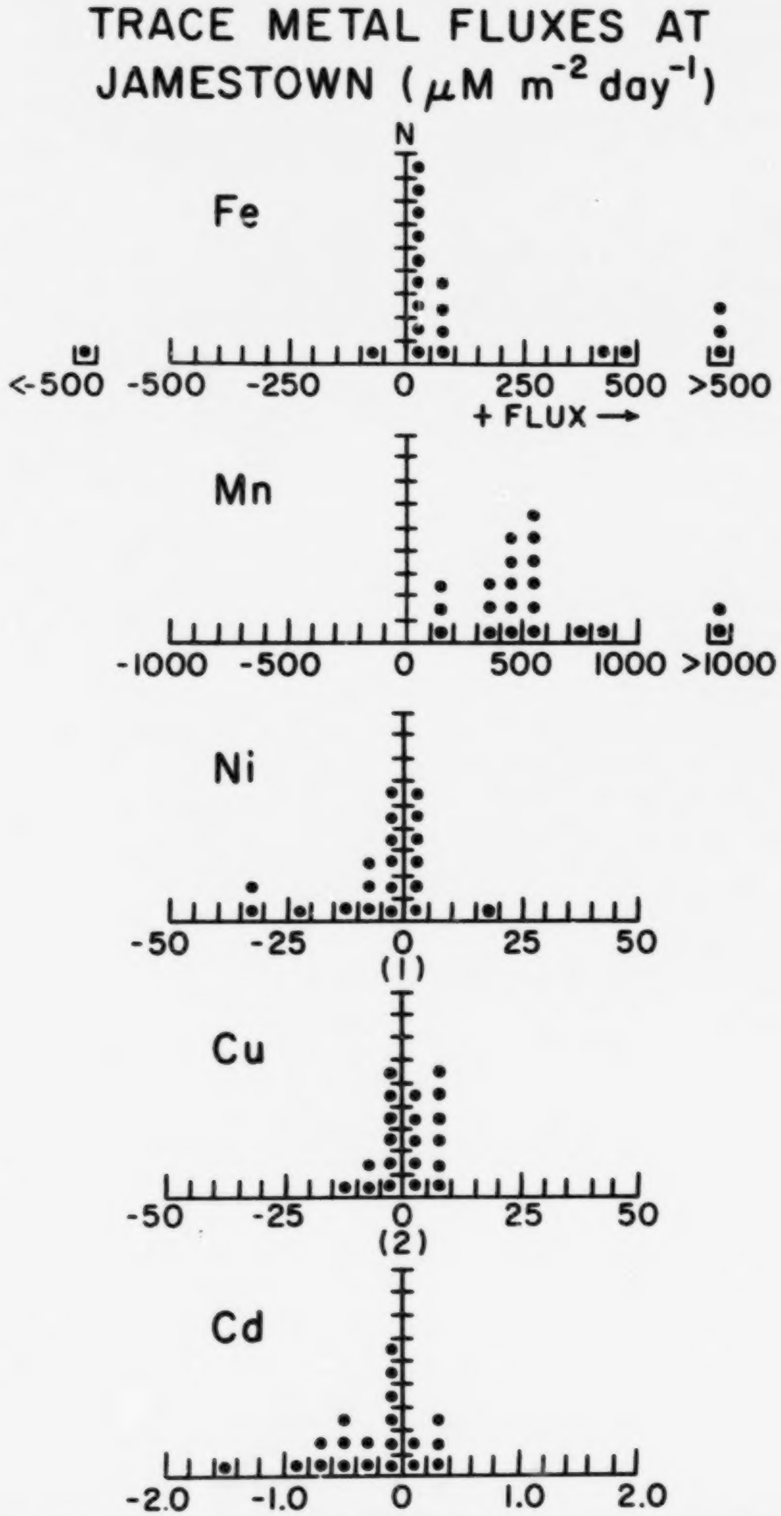

Figure 2-4. Histograms of heavy metal fluxes at Jamestown North (units are $\mu\underline{M}$ m^{-2} day^{-1}).

Table 2-4. Benthic Fluxes Measures at the Jamestown North Study Site and Estimate Doubling Times for Cu, Ni, Mn and Fe in Narragansett Bay

	Mean flux and standard deviation ($\mu g\ cm^{-2}\ day^{-1}$(1)	Concentration of dissolved metal in Narragansett Bay (ppb)	Time for benthic flux to double water column concentration (days)
Cd	-0.0029±0.0043	0.10	>71
Cu	-0.009±0.044	2.0	>57
Ni	-0.035±0.064	3.0	>100
Mn	2.1±0.8(2)	10	5
Fe	0.17±0.23		

NOTES:

(1) Based on twelve determinations.

(2) Calculated excluding one anomalously high value believed to reflect contamination.

summertime, release of manganese from sediments is a major source of manganese in Narragansett Bay, but release of nickel, copper and cadmium are probably not significant.

TRACE METAL BUDGETS IN NARRAGANSETT BAY: Mn AND Cu AS EXAMPLES

The preceeding discussion suggests that benthic fluxes will be a source of Mn, but not Cu, to the waters of Narragansett Bay. The distribution of these metals in the Bay provides a check on these conclusions, as will be seen from the following discussion.

Graham et al (6) did a mass balance for dissolved and particulate manganese in Narragansett Bay in the Summer of 1973. Their results for the distribution of dissolved manganese in the main part of the Bay are shown in Figure 2-5. It is readily seen that dissolved manganese is not a single-valued function of salinity: at a given salinity, surface waters have far lower manganese concentrations than deep waters. The manganese concentration of surface samples is far less than that expected from a simple conservative mixing model in which the deep water concentration is approximately equal to the conservative concentration. Graham et al (6) interpreted these results as indicating that manganese is scavenged (presumably by oxidation and precipitation) throughout the waters of the estuary, and bottom waters are enriched relative to surface waters by the benthic flux of manganese.

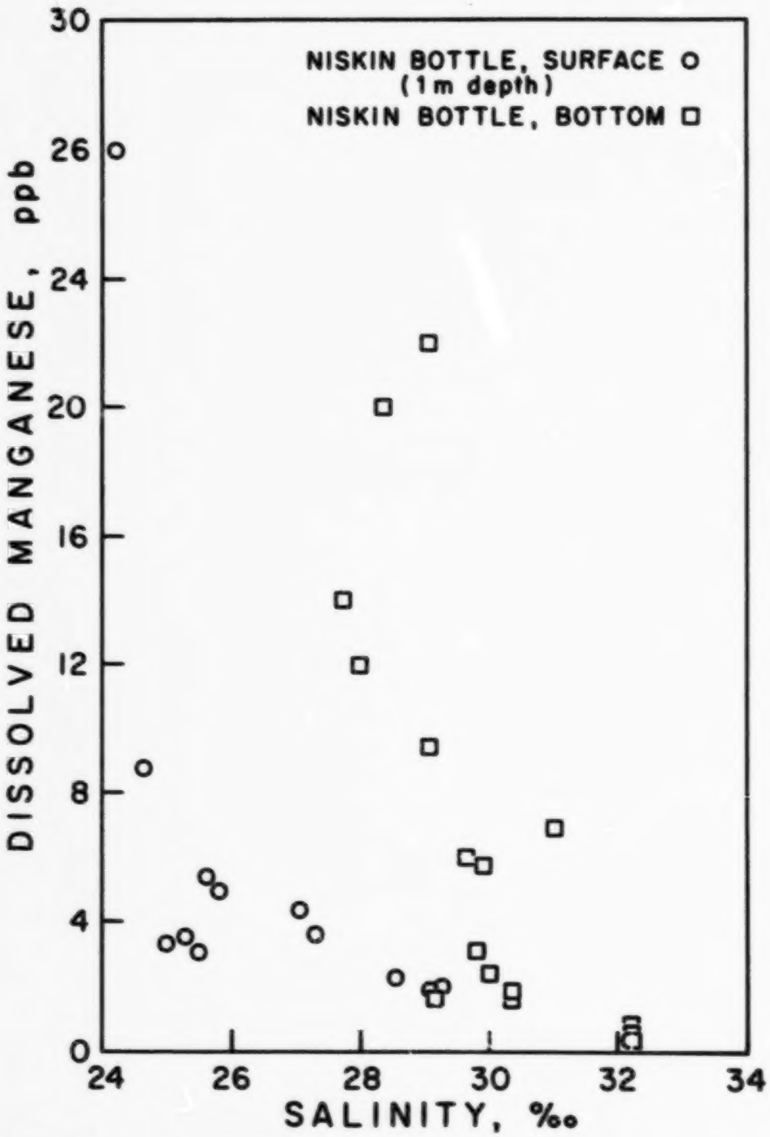

Figure 2-5. Dissolved Mn vs. salinity in Narragansett Bay, in the Summer of 1973.

Dissolved copper and manganese (along with O_2, nutrients and other metals) were measured in the waters of Narragansett Bay in the Spring of 1977 by the Narragansett Bay Study Group (in preparation). At this time of year, manganese was found to be nearly conservative, showing no surface water depletion and only a small deep water enrichment. The contrast between manganese behavior in the spring and summer probably reflects slower oxidative precipitation, slower benthic fluxes, and more rapid flushing of the Bay under springtime conditions of lower temperatures and higher runoff.

The dissolved copper distribution in Narragansett Bay in the springtime of 1977 is shown in Figure 2-6 (results of the Narragansett Bay Study Group). Concentrations were determined using the methods outlined earlier for pore waters. Several copper values fall far above a conservative mixing line, and are believed to reflect contamination. Most values, however, appear to define a simple conservative mixing line. These results show no evidence for an input of copper into the Bay by diffusion out of sediments. The Bay water results will be discussed in more detail elsewhere.

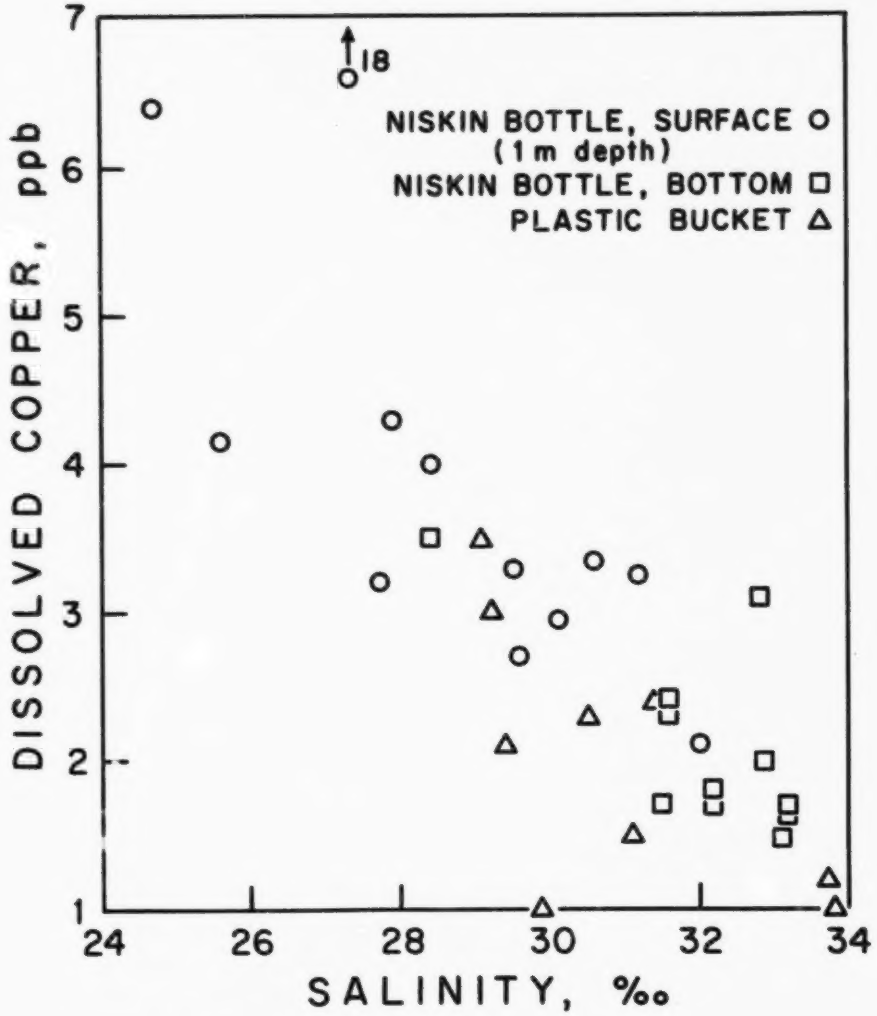

Figure 2-6. Dissolved Cu vs. salinity in Narragansett Bay, in the Spring of 1977 (Narragansett Bay Study Group, in prep.).

SUMMARY

The results summarized here are consistent with the hypotheses of earlier workers that metals forming highly insoluble sulfides will be sequestered in anoxic marine sediments.

Our conclusions reflect results of a limited study on a small number of metals during one or two seasons in a single estuary. The conclusions are thus preliminary, and cannot be extrapolated to other seasons, metals or estuaries. Organic complexing, in particular, may render certain heavy metals far more soluble than would be expected from sulfide solubilities calculated, considering inorganic ion pairing only.

ACKNOWLEDGMENTS

We are grateful to Scott Nixon, Candace Oviatt and colleagues, for their generous cooperation in collecting samples, and for the loan of sampling equipment. We also wish to express our appreciation to Nile Luedtke, who participated extensively in the pore water and benthic flux determinations, and scuba divers Paul Benoit, George Morrison, Allen Myers and Bob Pavia for their skill and care in obtaining *in situ* benthic flux samples. This work was supported by a grant from the Environmental Research Laboratory of the Environmental Protection Agency.

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THE USE OF INTRODUCED SPECIES (*MYTILUS EDULIS*) AS A BIOLOGICAL INDICATOR OF TRACE METAL CONTAMINATION IN AN ESTUARY

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The use of introduced as well as indigenous marine species as biological monitors or indicators of water quality is being evaluated at the Environmental Research Laboratory, Narragansett (ERLN). This paper presents data that demonstrate the edible blue mussel, *Mytilus edulis*, to be an effective indicator of metal pollution when introduced along a gradient of anthropogenic stress. *M. edulis* were collected from a commercially-fished mussel bed in Narragansett Bay, Rhode Island, and held in a laboratory seawater system for six days. Sub-groups were deployed in polluted and clean sections of that estuary, respectively, for a period of four weeks.

Atomic absorption analyses revealed that *M. edulis* from the polluted section had significantly higher levels of lead, nickel, and copper when compared to *M. edulis* from the clean part of the estuary and those retained in the seawater system at the laboratory as controls. No differences were apparent between the three groups in the case of cadmium, chromium, vanadium, and zinc; however, comparisons between introduced *M. edulis* and indigenous *Mercenaria mercenaria*, demonstrated *M. edulis* to be an effective surrogate biological monitor for *M. mercenaria* in the case of lead, nickel, and copper.

INTRODUCTION

The area of study is Narragansett Bay, Rhode Island, U.S.A. (Figure 3-1). The Bay has been described as "abnormally stressed" by man's activities in its upper reaches, and as being divisible into a polluted upper Bay, a transitional zone, and a lower Bay having water of high quality (1). Bottom water salinities range 28-31^{o}/oo in the upper reaches and 30-32^{o}/oo in the lower Bay. Temperature seasonally escalates from -1°C to 26°C in various sections of the estuary.

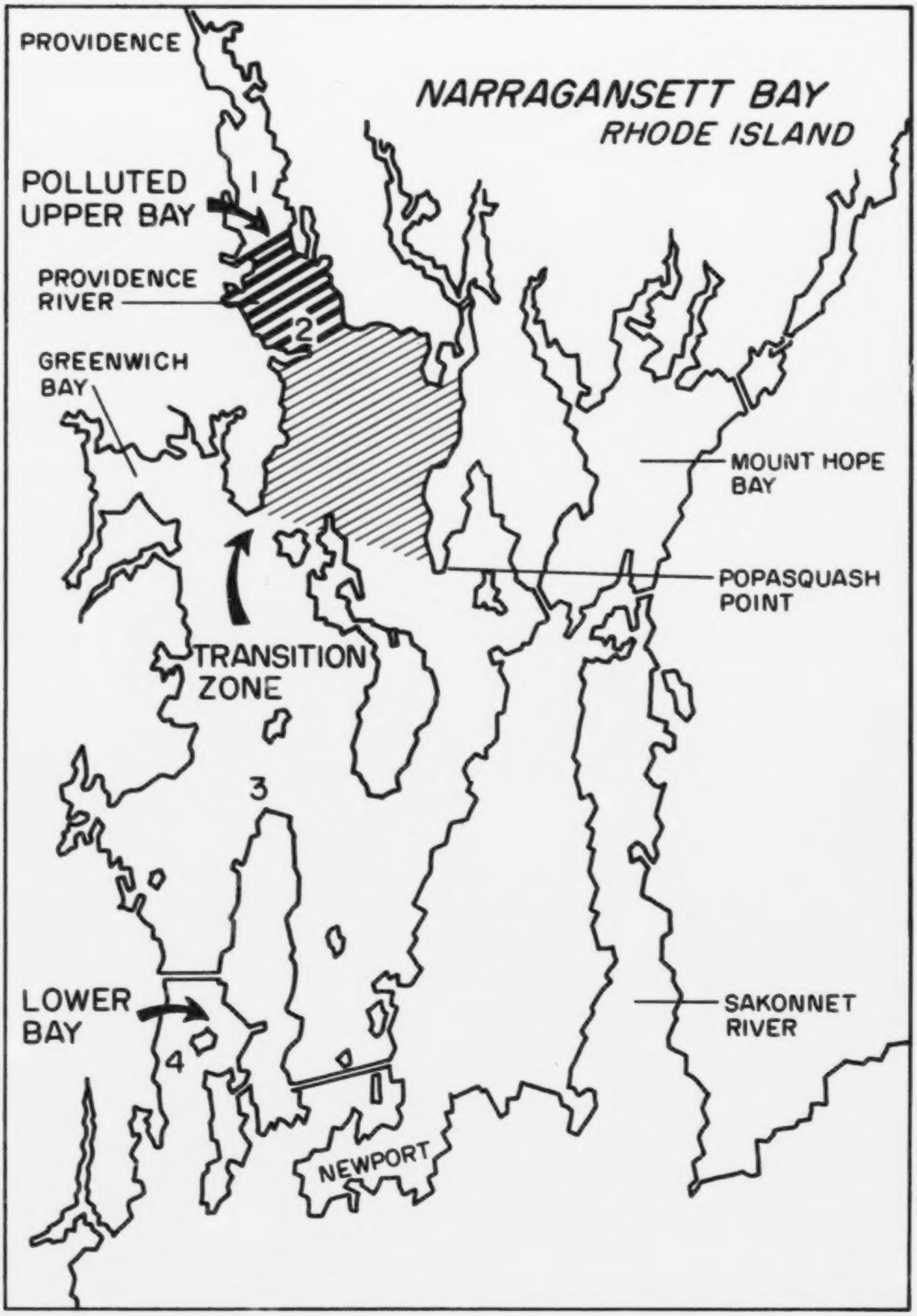

PROVIDENCE
NARRAGANSETT BAY
RHODE ISLAND
POLLUTED UPPER BAY
1
PROVIDENCE RIVER
2
GREENWICH BAY
MOUNT HOPE BAY
POPASQUASH POINT
TRANSITION ZONE
3
LOWER BAY
4
SAKONNET RIVER
NEWPORT

Figure 3-1. Area of study.

BEST DOCUMENT AVAILABLE

Major environmental differences within the system are attributable to a history of pollution effects in the upper reaches of the Bay (Figure 3-1). Major sources of pollution are domestic waste treatment plants, which include industrial effluents from such activities as metal plating and jewelry manufacturing, and urban runoff.

Fine sediments in the upper area (Stations 1 and 2) are anaerobic, characteristically having the redox boundary at the sediment-water interface, as well as having a strong odor of H_2S. In the lower Bay (Stations 3 and 4), fine sediments have a well-defined aerobic layer with a redox boundary defined between 5 and 10 cm below the sediment-water interface (2). Metals in upper Bay sediments include typically elevated levels of zinc (337 ppm), lead (167 ppm), copper (493 ppm), and chromium (208 ppm) compared to lower Bay levels of zinc (119 ppm), lead (40 ppm), copper (48 ppm) and chromium (53 ppm) (2). In addition, higher concentrations of hydrocarbons have been reported in upper Bay sediments compared to levels found in the lower Bay (3). Over the past few years, a transect of stations, indicated in Figure 3-1 as 1, 2, 3, and 4, has been used to study the effects of pollution from north to south in the Bay.

M. mercenaria is a molluscan species indigenous to all areas of the Bay. Phelps and Myers (4) compared levels of aluminum (Al), cadmium (Cd), cobalt (Co), copper (Cu), lead (Pb), manganese (Mn), nickel (Ni), silver (Ag), titanium (Ti), vanadium (V), and zinc (Zn) between *M. mercenaria* collected from the "polluted" upper Bay and the "clean" lower Bay. Of particular interest is the fact that after a thirty-day period of depuration, *M. mercenaria* from the upper polluted part of the Bay retained significantly higher levels of Cd, Cu, Ni, Pb, and Ti compared to *M. mercenaria* collected from the lower "clean" part of the Bay (Figure 3-2).

This paper reports on the use of *M. edulis* as an *introduced* biological monitor for trace metals. The specific goals of the study were:

1. To observe whether or not introduced *M. edulis* bioaccumulation would reflect the spatial differences in trace metal levels previously observed in sediments and indigenous *M. mercenaria.*

2. If such differences were reflected by *M. edulis*, over what time frame were differences observable?

3. If quantitative differences were reflected, how do metals accumulated by introduced *M. edulis*, compare qualitatively to metals accumulated by indigenous *M. mercenaria*?

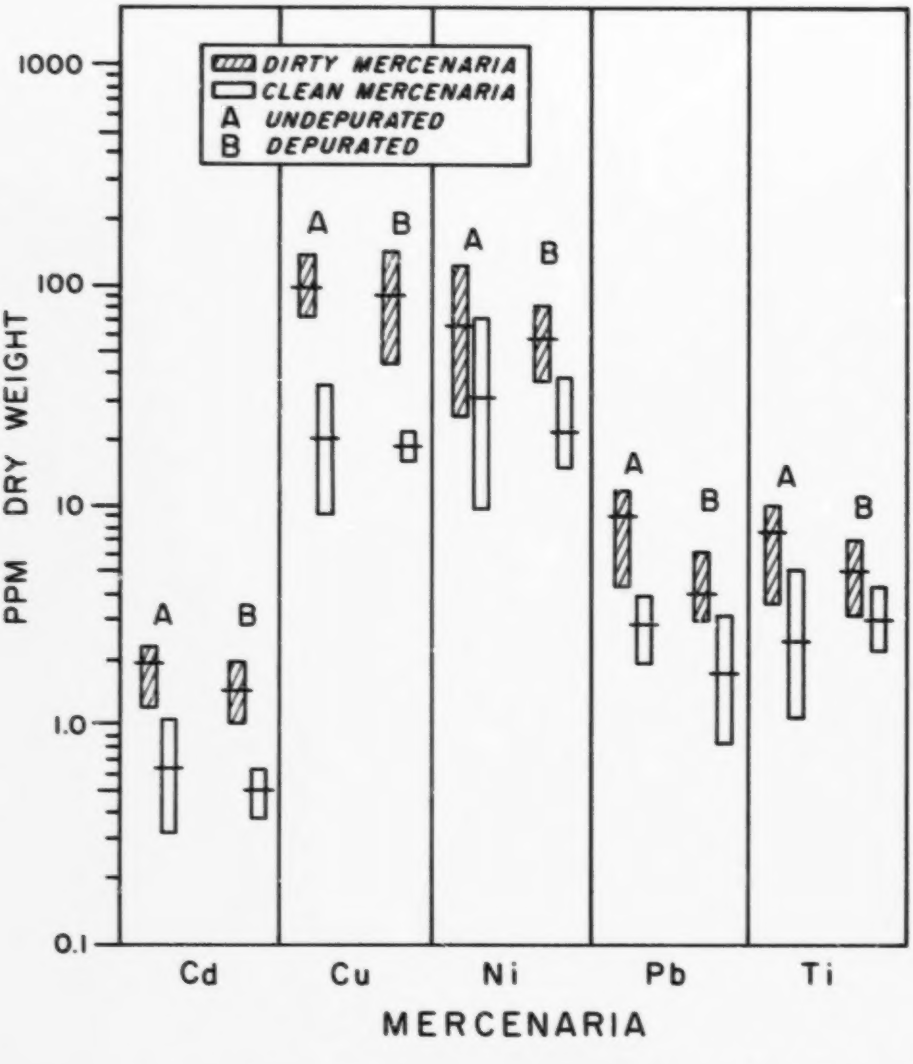


Figure 3-2. Metal levels in indigenous Mercenaria mercenaria from clean and polluted parts of Narragansett Bay, RI.

Note: Range shown is one standard deviation on either side of the mean.

The mussels used in this study were collected at one time immediately south of Popasquash Point, Narraganasett Bay, R.I. (Figure 3-1), and held in a laboratory flow-through system for six days prior to the first deployment.

Field Methods

Since metals were chosen as the initial pollutants of interest, a completely metal-free apparatus was desired for deploying mussels in the field. In view of potential use by other investigators, a simple, relatively inexpensive apparatus which was easy to deploy and service was a secondary goal of the design process. The present apparatus is shown in Figure 3-3.

A plastic float is attached to a concrete weight by approximately two meters of polypropylene line. The mussel holding baskets, 15 x 15 x 15 cm polypropylene test tube baskets with snap-on lids, are suspended from the line approximately 1 meter above the sediment surface.

A *M. edulis* monitoring station is deployed in a two-step operation. The apparatus, minus the mussel baskets, is lowered to the bottom in 5 to 8 m of water, after which typically eight mussel baskets are taken down and suspended in groups of four from the line by scuba divers. The deployment operation requires 10 to 15 minutes on-station and subsequent sampling only about five minutes. Each basket of 20 mussels serves as a subsample, thereby allowing removal of the desired sample without disturbing the remaining mussels. Samples for metals analyses are immediately transferred to "ziploc" polypropylene bags and frozen until analyzed.

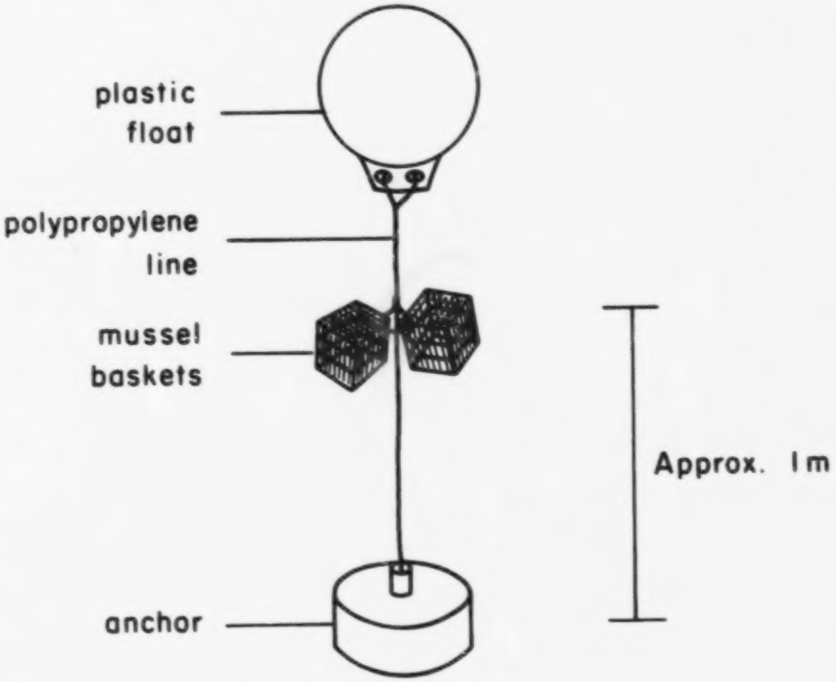


Figure 3-3. Schematic of subsurface mussel station.

The first collection was made from each station after an exposure period of three weeks. A second collection was made one week later.

Analytical Methods

The mussel tissue is analyzed for metal content by flame Atomic Absorption Spectrometry after wet digestion in concentrated nitric acid. Each sample is oven dried to constant weight, then digested in concentrated nitric acid in a simple reflux system. The digestate is filtered on transfer to 50 ml volumetric flasks, brought up to volume, and analyzed on a Perkin-Elmer Model 603 Atomic Absorption Spectrophotometer using Deuterium arc background correction where necessary. The raw data are reduced to ug of metal/gram of tissue, on both a wet and dry weight basis, by computer.

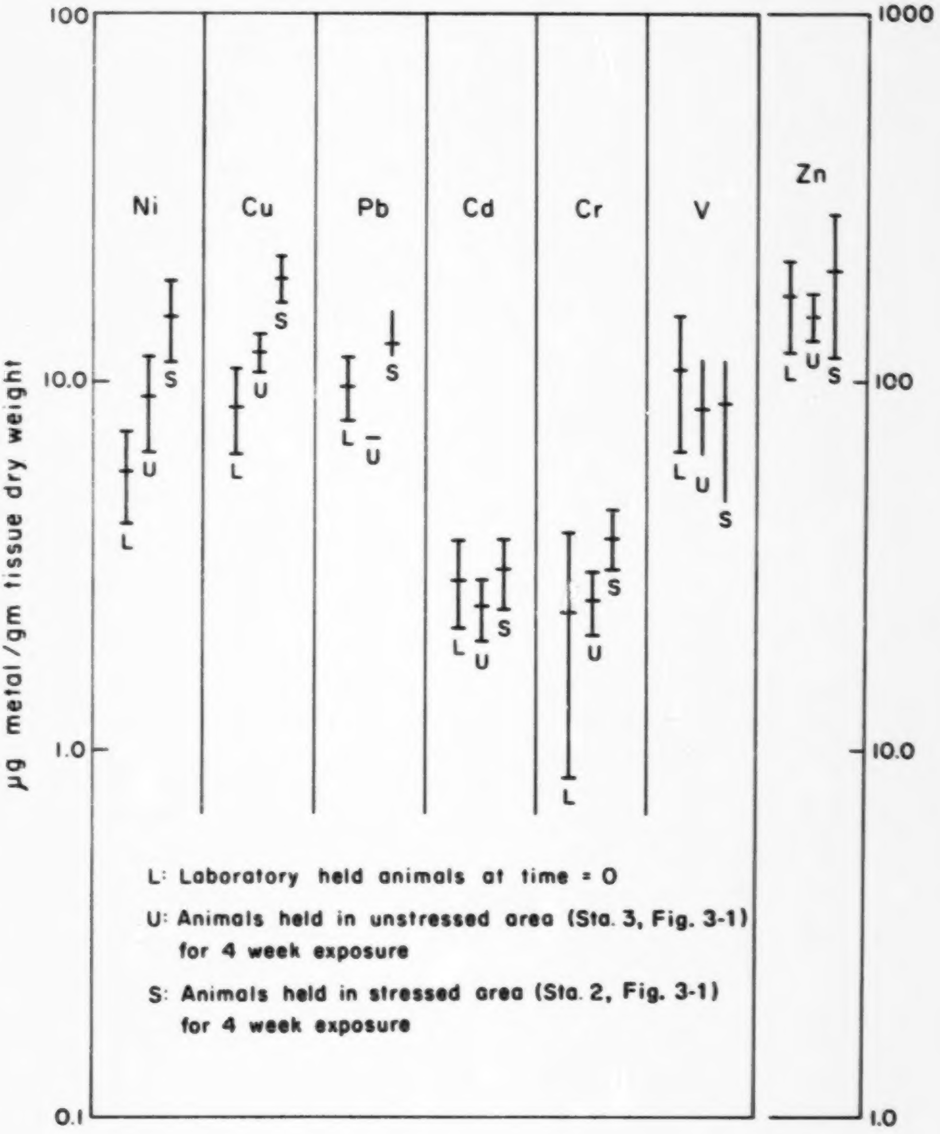
RESULTS

Results of analyses for Cd, Pb, Ni, Cu, chromium (Cr), V, and Zn on *M. edulis* from Stations 2 and 3, as well as from controls, are presented in Figures 3-4 and 3-5 and Table 3-1.

Except in the case of Pb and V, where the numbers of samples having detectable limits were below the minimum number required for the statistic, the standard deviation is comparable between a sample of 41 or 10 (Table 3-1). This fact establishes that 10 *M. edulis* are a reasonable sample size with the noted exceptions of Pb and V. After four weeks, Cd levels in *M. edulis* from mid-Bay, Station 3, were slightly lower than either laboratory-held *M. edulis* or those collected from the polluted area at Station 2. However, the overlap in Cd values between the three sites renders differences insignificant (Figure 3-4).

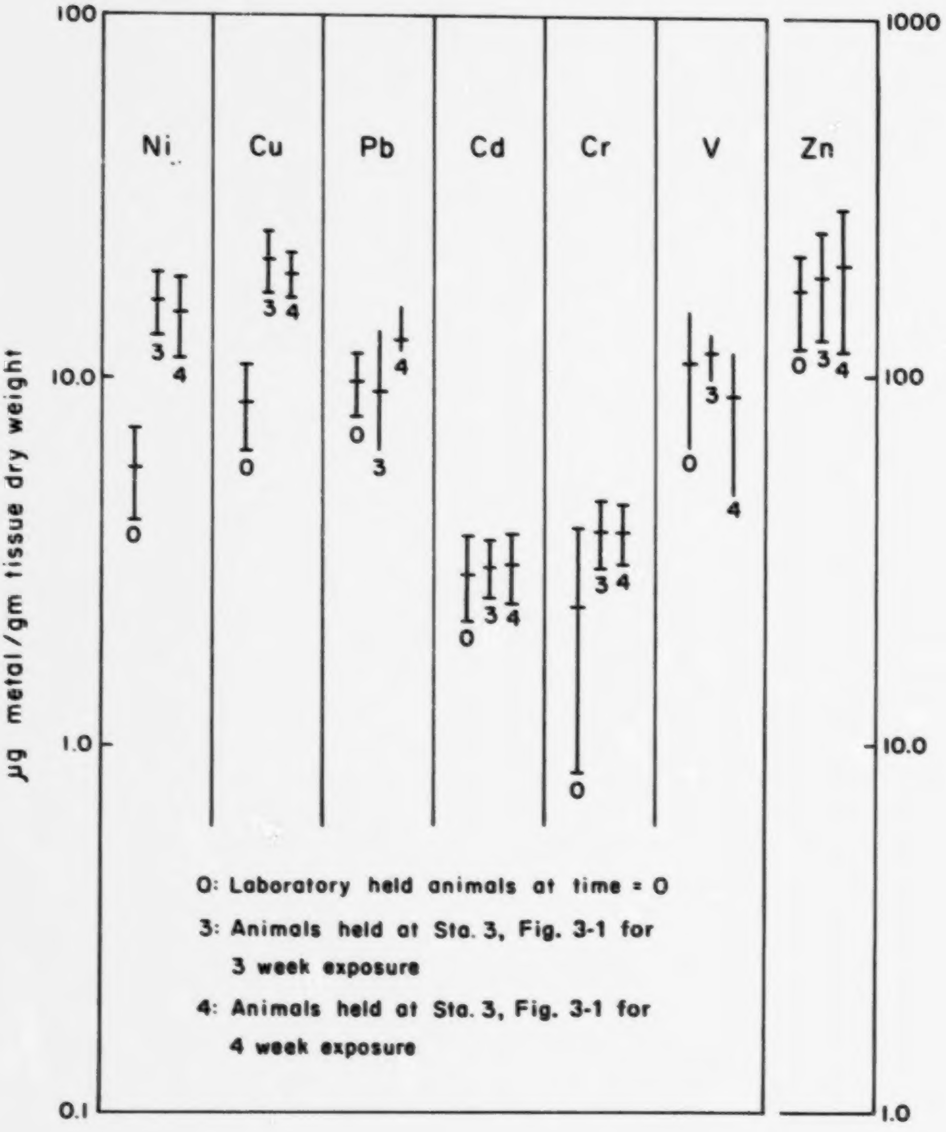
Lead, in those four animals from the polluted area having detectable levels, was higher than those levels detected in the laboratory-held animals, and the single animal having detectable levels from Station 3. However, due to the low number of sampling points, statistical significance cannot be established for these data (Figure 3-4).

Nickel levels in *M. edulis* from Station 2 are significantly higher than those measured in laboratory-held animals. *M. edulis* from Station 3, while not being significantly different from Station 2, or laboratory-held animals, clearly fall in a range midway between those polluted and clean areas. A gradient of Ni, with highest levels in the polluted area of the Bay, diminishing in the mid-Bay, having lowest levels in the lower Bay, is demonstrated by these data. It is of interest to note that only 19 of the 41 laboratory-held animals had detectable levels of Ni.



Note: Bar lines, when present, indicate one standard deviation on either side of the mean; otherwise total range is indicated.

Figure 3-4. Metal levels in Mytilus edulis introduced into stressed and unstressed environments, Narragansett Bay, RI, September-October, 1976.



Note: Bar lines, when present, indicate one standard deviation on either side of the mean; otherwise total range is indicated.

Figure 3-5. Metal levels in Mytilus edulis indicating uptake from time=0 to time=4 weeks, Narragansett Bay, RI, September-October 1976.

Table 3-1. Metal Levels in *Mytilus edulis*

	Cd	Pb	Ni	Cu	Cr	V	Zn
Lab-held	2.89	9.7	5.7	8.5	2.35	10.7	170
41 specimens	.78	1.9	1.6	2.2	1.52	4.3	52
(time = 0)	(40)	(10)	(19)	(41)	(41)	(22)	(41)
Stressed	3.04	12.6	15	19	3.76	10.5	199
10 specimens	.65	10.8-15.5	3.69	2.8	.69	4.7-11.4	83
(time = 4 weeks)	(10)	(4)	(10)	(10)	(10)	(5)	(10)
Unstressed	2.42	7	9.1	12	2.53	8.4	149
10 specimens	.47	7	2.7	1.4	.50	6.4-11.5	22
(time = 4 weeks)	(10)	(1)	(10)	(10)	(10)	(5)	(10)

Metal levels in *Mytilus edulis* expressed above in ug/g dry weight as:

Mean
Standard deviation or range
(no. of organisms above detection limit)

Copper levels in *M. edulis* are significantly higher in that group of animals from polluted Station 2 than either Station 3 or laboratory-held animals. However, the latter two animal groups have statistically similar levels. Copper data show a sharp drop from the polluted area to the levels in both lower Bay groups (Figure 3-4).

Chromium levels in *M. edulis* from the three groups are not significantly different (Figure 3-4).

Vanadium levels were above detection in only about one-half of the individuals collected from each station. While the data are too sparse for statistical analysis, no difference between the levels at the three stations is apparent (Figure 3-4).

Variability in levels of Zn is so great within each station that meaningful comparison between stations is not possible.

After a three-week period, *M. edulis* from polluted Station 2 had accumulated significantly higher levels of Ni and Cu than had *M. edulis* from the clean Bay stations (Figure 3-5). Similarly, Station 2 values after three weeks were significantly higher than values from control animals sacrificed at time zero. However, levels remained the same in all animal groups after one additional week of exposure.

DISCUSSION

Currently, *M. edulis* is being used as an indigenous biological monitor for a variety of materials including petroleum hydrocarbons, chlorinated hydrocarbons, and transuranics, in addition to trace metals in coastal waters of the United States (5). Similar activity is underway in the United Kingdom and Germany as well. Because of its ubiquitous distribution, *M. edulis* is being considered as an international monitoring organism.

To date, the use of *M. edulis* appears to be limited to that of an indigenous monitor. We are not aware of an approach similar to that reported here where *M. edulis* is used as an introduced biological monitor.

The results indicate that *M. edulis* as an introduced species does reflect elevated levels of metals previously observed in the polluted section of Narragansett Bay, in the sediments and the indigenous mollusc, *Mercenaria mercenaria*. Nickel and copper were concentrated to significantly higher levels in *M. edulis* introduced into the polluted section of the Bay compared to those introduced in the clean lower Bay, and those held in our laboratory flow-through system. Lead levels were observed to be higher in animals from collecting Station 2, than in either collecting Station 3 or the laboratory; however, statistical significance was not established due to the small data set. Cadmium, chromium, vanadium, and zinc were not reflected at higher levels in *M. edulis* from the polluted area.

With one exception, that being in the case of Cd, these results reflect those reported by Phelps and Myers (4) for *M. mercenaria* collected in the same parts of Narragansett Bay. In that study, *M. mercenaria* from the polluted area were shown to concentrate to higher levels, but not depurate, Cd, Pb, Ni, and Cu compared to lower Bay animals. No differences were observed in levels of V and Zn between the two groups of *M. mercenaria* before or after depuration. Thus, *Mytilus edulis*, when used as an introduced monitor, is demonstrated to reflect metal levels observed in the major resident or indigenous species in three out of the four metals of note.

Higher metal accumulations were established after three weeks of monitoring by *M. edulis* introduced in the polluted section of the Bay for Ni and

Cu. Higher levels of Pb appeared after four weeks of exposure. The relatively quick response time established in the case of Ni and Cu represents an obvious advantage to the use of *M. edulis* as a biological monitor. For these metals, *M. edulis* quickly reflects a situation which effects the long-lived indigenous species, *M. mercenaria*.

These results encourage further study. Our three specific goals listed in the Introduction have been answered in the affirmative:

1. Introduced *M. edulis* from polluted areas reflect elevated levels of metals, as did sediments and *M. mercenaria* reported in previous work.
2. *M. edulis* displays a relatively short response time in accumulating elevated metal levels – three weeks in the case of Ni and Cu, and four weeks in the case of Pb.
3. *M. edulis*, the introduced biological monitor, took up three of the four metals previously demonstrated to have been accumulated and retained by the resident species, *M. mercenaria*.

The results reported here are based on data collected when the annual temperature cycle was declining toward winter levels. This fact may account for the leveling-off of Ni and Cu observed between weeks three and four at Station 2.

Further studies along the transect in Narragansett Bay, and in other comparable areas, including a complete annual temperature cycle, are being carried out to supplement the knowledge gained in this study on the use of *M. edulis* as an introduced biological indicator of man's impact on the environment.

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TRACE METAL SPECIATION AND TOXICITY IN PHYTOPLANKTON CULTURES

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ABSTRACT

The toxicity of trace metals to phytoplankton has been demonstrated to depend on metal ion activities. The various chemical processes that control metal speciation, and thus activities in aquatic systems, are inorganic complexation, chelation, precipitation and adsorption. For example, the activity of metals such as mercury, cadmium or lead are controlled in saline waters of low organic content by the formation of chloride and bromide inorganic complexes. For mercury, this is also the case in typical phytoplankton culturing media. Artificial chelating agents permit convenient manipulation of metal ion activities in algal toxicity experiments. However, kinetic phenomena can result in transient peaks in metal ion activities and lead to large overestimations of toxicity. The release of metal complexing agents by algae is not expected, in general, to affect markedly the chemistry of metals in highly chelated artificial media except in cases of high specific affinity. The greatest complication in interpretation of phytoplankton toxicity experiments arises from the presence of solids in the culture medium. These can precipitate during the preparation of the medium, or as a result of the pH increase due to photosynthetic carbon uptake. The kinetics of precipitation of these solids, their aging and the adsorption of trace metals on their surface, lead to variations in metal activities that are difficult to quantify, and do not permit proper assessment of the toxic effects. Understanding the global aquatic chemistry of trace metals in algal culture media, is a *sine qua non* prerequisite to proper design and interpretation of toxicity experiments.

INTRODUCTION

Using copper as the principal example, this study aims at establishing a chemical framework for the study of laboratory and natural processes involving trace metal toxicity to phytoplankton. If it is a reasonable assumption that the mechanisms of metal toxicity are similar in many phyla, this framework should be useful for toxicity studies with many aquatic organisms, and can serve as the general basis for design and interpretation of such studies.

Although the importance of both organic complexing agents and trace metals in phytoplankton cultures has been recognized for some time, the critical role played by the speciation of trace metals in controlling their toxicity and availability to algae has just begun to be understood. It has now been established that it is the activity of the free ions, rather than the total metal concentrations, which determine the toxicity of metals to phytoplankton (46, 2). The study of the chemical processes which govern the activity of a given trace metal in culture media becomes then a prime area of concern to phycologists interested in metal toxicity experiments. An enumeration of these processes includes inorganic complexation, chelation, precipitation and adsorption. In addition, indirect chemical effects involving several interacting chemical species in the medium can influence trace metal activities in unobvious ways. In this study, these basic principles of Aquatic Chemistry (43) that apply to metals in phytoplankton cultures will be discussed systematically.

There are but a few free metal activities that can be experimentally measured in the range and under the conditions of interest. There are also few metallic complexes which can be analytically determined in chemical systems as complex as culturing media. Therefore, theoretical equilibrium calculations will be used throughout this paper to assess metal speciation and activities. The assumption of equilibrium is a reasonable one when the proper precautions are taken during medium preparation. Thermodynamic calculations provide, then, a convenient means of illustrating the critical chemical principles, even if they have inherent uncertainties. Complications introduced in the chemistry of the system by kinetic phenomena, or by the influence of the algae, will be discussed for each of the examined processes.

Copper has been the metal of choice in studies of metal toxicity of phytoplankton because it has been postulated that cupric ion toxicity might play a role in the ecology of phytoplankton in some natural waters (6, 37, 7, 10). In keeping with this situation, this paper will focus, albeit not exclusively, on copper which provides a rather good example for metal speciation and toxicity in phytoplankton cultures.

EQUILIBRIUM SPECIATION OF METALS IN CULTURING MEDIA

Before studying in detail the role of chemical processes in controlling metal speciation, it seems useful to examine what metal species are expected to be important in typical culturing media.

In artificial media where the analytical concentrations of the components are precisely known, the exact composition of the system, including all soluble and insoluble species of the various metals and their activities, can be computed if a state of equilibrium or partial equilibrium is established (43). It should be underlined that such calculations of chemical speciation depend on correct identification of the principal species, and knowledge of the corresponding equilibrium constants. The calculations presented here were performed with the computer programs, REDEQL (25, 21) and MINEQL (49) which contain a list of possible species, and a selection of the necessary thermodynamic constants from a variety of sources (36, 38, 33).

Results of chemical equilibrium computations of three media recipes, the freshwater medium WC (11) and the seawater media F/2 (12) and Aquil (26), are shown in Table 4-1. Possible adsorption processes are not considered in these calculations. Note that some heavy metals (Pb, Cd, Hg, Ni, Co, Cr) which are not part of the recipes, have been added in trace amounts (10^{-9}M) to illustrate how they would be speciated if they were present as contaminants in the media. Such low metal concentrations affect the rest of the chemical system negligibly. Heavy metal speciation in all media is completely dominated by the chelation with ethylenediaminetatraacetate (EDTA) which is included in the recipes for the very purpose of chelating metals. An important exception is mercury, which, according to the computations, is present entirely as chloride complexes in F/2 and Aquil and half as hydroxide species in WC. For all metals, the free ion activities are several orders of magnitude smaller than their total concentrations. In all media, iron and manganese oxides and calcium phosphate (hydroxylapatite) are computed to precipitate at equilibrium. Calcium carbonate (calcite) is also shown to be saturated in the seawater media. Actual precipitation of these various solids is dependent on kinetic processes as will be discussed later.

The trace metal chemistry of such culturing media can be grossly affected by the presence of algal cells due to metal uptake. For example, typical values for the uptake of copper by phytoplankton are in the range 10^{-16} to 10^{-14} moles/cell (44, 35, 16). With the algal densities and the copper concentrations commonly used, a sizeable part of the total concentration of copper in the medium can thus be taken up by the cells. This underlines the necessity of "buffering" metal ion activities in toxicity experiments in order to render these activities relatively insensitive to total metal concentrations. The use of various chelating agents for this purpose will be discussed later.

INORGANIC COMPLEXATION

It has been observed in several instances that the toxicity of metals such as lead, cadmium, mercury or silver to a variety of organisms, from bacteria to

Table 4-1. Equilibrium Trace Metal Speciation in Typical Algal Growth Media

	WC(EDTA= $1.2\ 10^{-5}$ M)			F/2(EDTA= $1.2\ 10^{-5}$ M)			Aquil (EDTA = 5×10^{-6} M)		
	Analytical Concentration M	Computed Activity of free ion. M (-log)	Major Species	Analytical concentration M	Computed Activity of free ion. M (-log)	Major Species	Analytical Concentration M	Computed Activity of free ion. M (-log)	Major Species
Iron	1.2×10^{-5}	21.5	FeEDTA 22% $Fe(OH)_3(S)$ 78%	1.2×10^{-5}	21.5	$Fe(OH)_3(S)$ 99%	4.5×10^{-7}	21.5	FeEDTA 6% $Fe(OH)_3(S)$ 94%
Manganese	8.9×10^{-7}	14.7	$MnO_2(S)$ 100%	8.9×10^{-7}	14.7	$MnO_2(S)$ 100%	2.3×10^{-8}	14.7	$MnO_2(S)$ 100%
Copper	4.0×10^{-8}	14.8	CuEDTA 100%	4.0×10^{-8}	13.3	CuEDTA 99%	1.0×10^{-9}	14.4	CuEDTA 100%
Cadmium	1.0×10^{-9}	14.8	CdEDTA 100%	1.0×10^{-9}	13.3	CdEDTA 99%	1.0×10^{-9}	12.8	CdEDTA 98% $CdCl^+$ 1% $CdCl_2$ 1%
Zinc	7.9×10^{-8}	12.2	ZnEDTA 100%	7.9×10^{-8}	10.7	ZnEDTA 100%	4.0×10^{-9}	11.5	ZnEDTA 100%
Nickel	1.0×10^{-9}	16.8	NiEDTA 100%	1.0×10^{-9}	15.3	NiEDTA 100%	1.0×10^{-0}	14.8	NiEDTA 100%
Mercury	1.0×10^{-9}	20.4	HgEDTA 49% $Hg(OH)_2$ 51%	1.0×10^{-9}	23.6	$HgCl_4^{2-}$ 87% $HgCl_3^-$ 13%	1.0×10^{-9}	23.6	$HgCl_4^{2-}$ 87% $HgCl_3^-$ 13%
Lead	1.0×10^{-9}	16.3	PbEDTA 100%	1.0×10^{-9}	14.8	PbEDTA 100%	1.0×10^{-9}	14.3	PbEDTA 99%
Cobalt	5.0×10^{-8}	13.9	CoEDTA 100%	5.0×10^{-8}	12.3	CoEDTA 100%	1.0×10^{-9}	11.9	CoEDTA 99%
Chromium	1.0×10^{-9}	23.1	CrEDTA 100%	1.0×10^{-9}	21.5	CrEDTA 100%	1.0×10^{-9}	21.1	CrEDTA 100%

fish, decreases with increasing salinity of the water (45). One possible explanation for such a phenomenon is the decrease in metal ion activity resulting from the formation of ion pairs with the major anions of seawater. Figure 4-1 shows, for example, how the speciation of mercury and the activity of the mercuric ion vary in function of salinity in the medium F/2. As salinity increases the bromide complexes of mercury replace the EDTA chelate as the major species, followed by the chloride complexes as the salinity approaches that of seawater. In natural systems, in the absence of strong chelating agents, the same phenomenon would extend to other metals such as lead and cadmium. Table 4-2 illustrates this point by giving the major species of the various metals in Aquil where EDTA has been reduced to 10^{-8}M. Besides the chloride complexes, a number of carbonate (Cu, Pb), sulfate (Zn, Mn, Co) and hydroxide (Zn, Pb, Co, Cr) complexes become significant. Because the kinetics of formation of the various inorganic complexes of metals are typically fast (43), equilibrium is a good assumption in this instance, and the thermodynamic calculations should give accurate values of metal activities.

The role of carbonate complexation in decreasing the toxicity of metals in unchelated media has been verified for copper on *Daphnia magna* (3), for

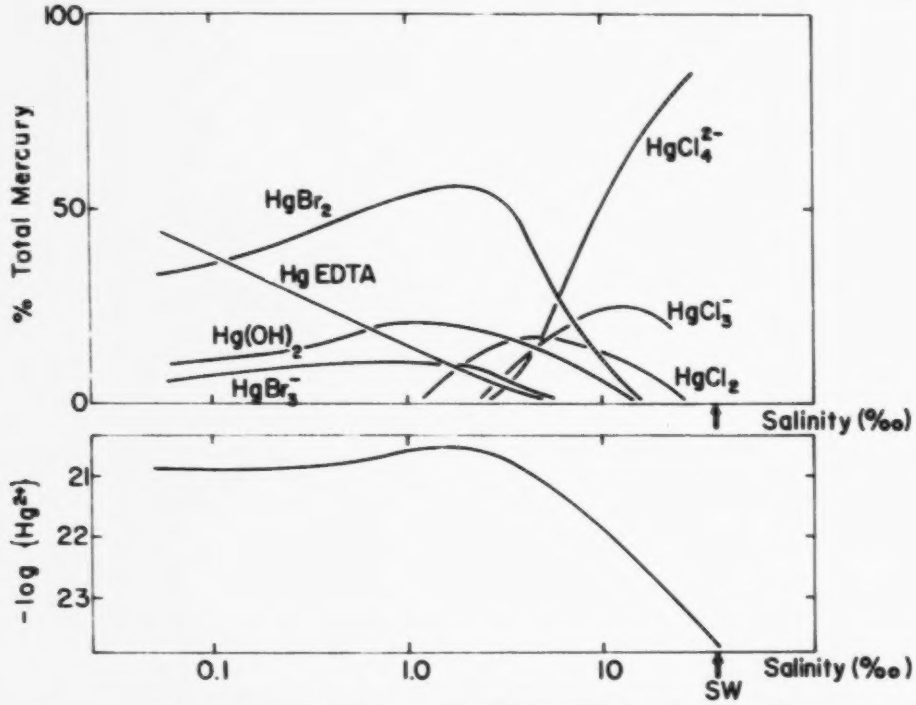

Figure 4-1. Chemical speciation of mercury in F/2 medium as a function of salinity at pH = 8.1.

Note: Top: mercuric complexes as a percent of the total mercury (10^{-9}M); Bottom: the activity of the mercuric ion. All other trace metals remain bound to EDTA throughout the salinity range. SW represents seawater, salinity 33‰.

Table 4-2. Speciation of Trace Metals in Aquil with EDTA Concentration Reduced to 10^{-8}M.

	Major Species	Percent of Total Metal
Iron	$Fe(OH)_3(S)$	100%
Manganese	$MnO_2(S)$	100%
Copper	Cu^{2+}	1%
	CuEDTA	87%
	$CuCO_3$	12%
Cadmium	Cd^{2+}	3%
	$CdCl_3^+$	39%
	$CdCl_2$	42%
	CdEDTA	6%
Zinc	Zn^{2+}	60%
	$ZnSO_4$	8%
	$ZnCl^+$	3%
	$ZnCl_3$	1%
	ZnEDTA	27%
	$ZnOH^+$	1%
Nickel	NiEDTA	99%
Mercury	$HgCl_4^{2-}$	87%
	$HgCl_3^-$	13%
Lead	$PbCO_3$	21%
	$PbCl_3^-$	50%
	$PbCl_2$	5%
	$PbCl^+$	3%
	PbEDTA	20%
	$PbOH^+$	1%
Cobalt	Co^{2+}	51%
	$CoSO_4$	11%
	$CoCl^+$	21%
	CoEDTA	15%
	$CoOH^+$	2%
Chromium	CrEDTA	57%
	$Cr(OH)_4^-$	40%
	$Cr(OH)_2^+$	3%

copper on some fishes (31), and for cadmium on a grass shrimp (45). Although there has been no experiment reported to date that provides direct evidence for the importance of inorganic complexation in controlling metal toxicity to phytoplankton, this result can be inferred from data with these other organisms, and from the general demonstration that metal ion activities are the important parameters of toxicity to algae.

Through uptake of carbon dioxide for photosynthesis, algae can modify the inorganic species of metals by decreasing the total concentration of carbonate in the system and increasing the pH. As pH increases, the hydroxyl ion activity increases and so does the importance of metal hydroxide complexes. The effect on the carbonate ion activity and on the metal carbonate complexes is less straightforward, and depends on the original pH of the medium. In seawater media (pH 8), and in freshwater media around neutral pH, the carbonate complexes will increase with CO_2 uptake due to the predominance of the resulting pH increase over the total carbonate decrease. Such variations in metal chemistry can be alleviated by bubbling air in the cultures, thus insuring a steady concentration of carbonate in the medium.

CHELATION

The history of the development of artificial culturing media for algae is in part that of the replacement of "growth factors" and "soil extracts" by chelating agents (17). The exact role of these chelating agents in promoting algal growth has been a subject of some controversy (6, 14). It is now well established that they do control the toxicity of various heavy metals – copper in particular (46). Whether they also increase the availability of some metallic nutrients – chiefly iron – is yet unproven. Figure 4-2 shows the percentage of chelated metal and the metal activities in Aquil (with contaminant metals) as a function of the concentration of EDTA, by far the most widely utilized chelating agent in algal media. Note that the order in which the metals are chelated by EDTA is not simply related to either the metal ion activities or their affinities for EDTA (Fe>Cr>Cu>Ni>Pb>Zn, Cd, Co).

Other chelating agents which are commonly used include nitrilotriacetate (NTA), citrate and various amino acids. "Tris" (tris(hydroxymethyl)amino methane) commonly used as a pH buffer for biological experiments has received much use in recent studies of copper toxicity to phytoplankton (44). Used in conjunction with EDTA which chelates the other metals at a very low concentration, Tris permits a convenient manipulation of the cupric ion activity. Figure 4-3 illustrates this point by comparing how the cupric ion activity varies with total copper in Aquil (EDTA = $10^{-5.3}$M) and in a modified Aquil recipe containing Tris (EDTA = $10^{-6.3}$M and Tris = 10^{-3}M). Around $[Cu^{2+}] = 10^{-10}$M, where many toxicity studies are run, the cupric ion activity in the Tris medium is less sensitive to variations in total copper concentration than in the EDTA medium. However, with the proper precautions, both media yielded the same results in a study of copper toxicity to *Gonyaulax tamarensis* (2).

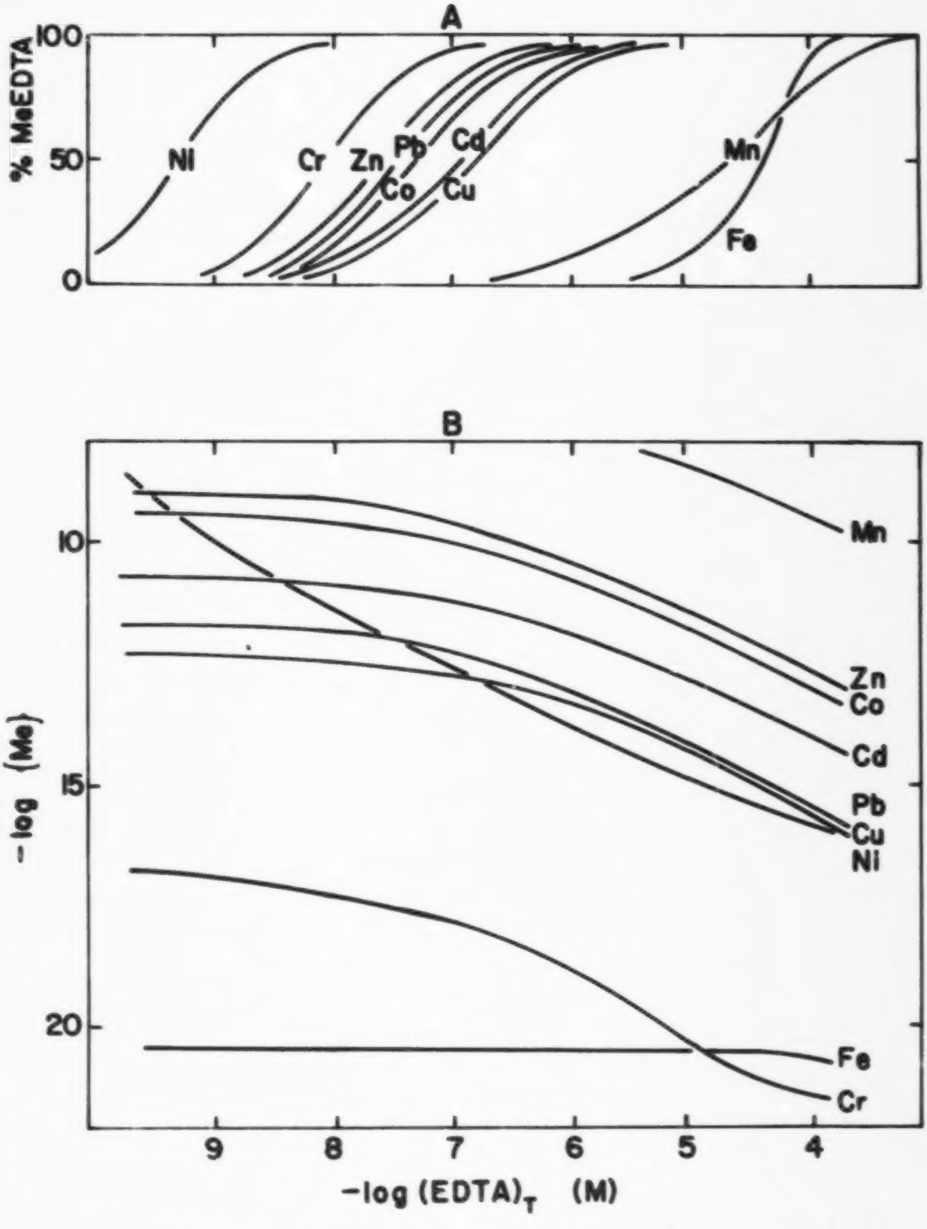

Figure 4-2. The effect of EDTA on the speciation of metals in Aquil.

Note: Total metal concentrations are given in Table 4-1, with Pb, Cd, Hg, Ni, Co, and Cr added as contaminants (10^{-9}M). A) The percent of each metal that is chelated (MeEDTA) versus total EDTA concentration, (M); B) Metal ion activity (M) versus total EDTA concentration (M).

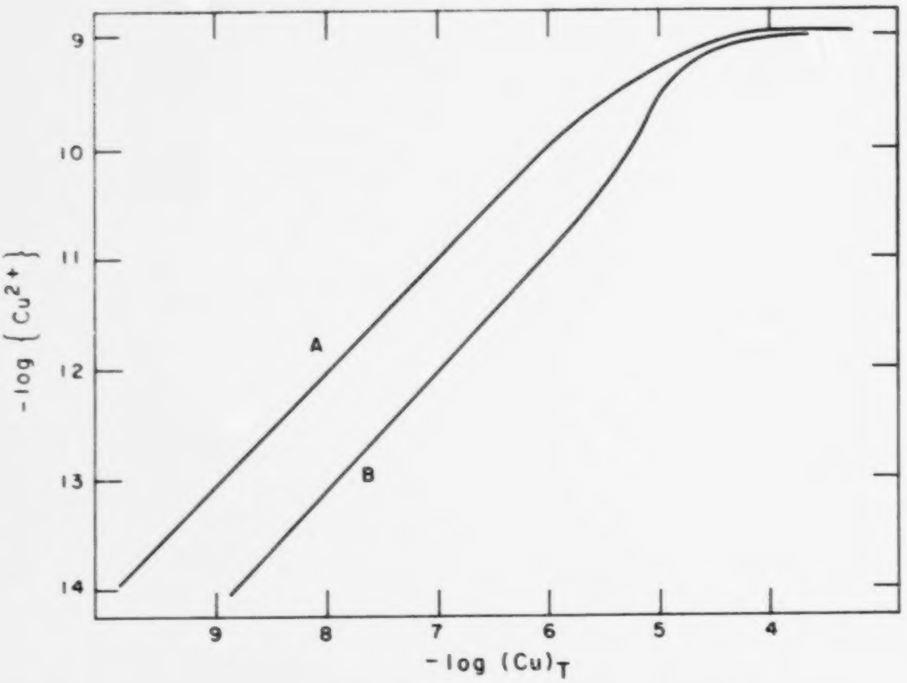

Figure 4-3. Computed activity of the cupric ion (M) versus total copper concentration for two Aquil recipes.

Note: Chelated with: A) $10^{-6.3}$M EDTA plus 10^{-3}M Tris and B) $10^{-5.3}$ EDTA.

Although the forward kinetic constants of chelate formation are invariably very large, resulting in quasi instantaneous kinetics in simple systems, the situation can be very different in systems as complex as culturing media. For example, when copper was spiked in Aquil cultures of *G. tamarensis*, a dramatic short term toxic response was observed much above that expected for the calculated equilibrium activity of the cupric ion (2). This phenomenon which was not observed when Tris replaced EDTA as the major copper chelating agent, was attributed to the slow kinetics of the metal exchange reaction:

$$Cu^{2+} + CaY \rightleftharpoons CuY + Ca^{2+}$$

This appears as a reasonable explanation, since the calcium chelate is the major form of EDTA in Aquil and the dissociation is slow. No such phenomenon can occur with Tris, whose major species in culturing media are the various protonated forms of the ligand. This can be checked directly by monitoring the cupric ion activity with a mixed sulfide electrode (34, 13) in chemical systems similar to the culturing media. Figure 4-4 presents the results of such an experiment, and leaves no doubt as to the slow kinetics of copper reaction with

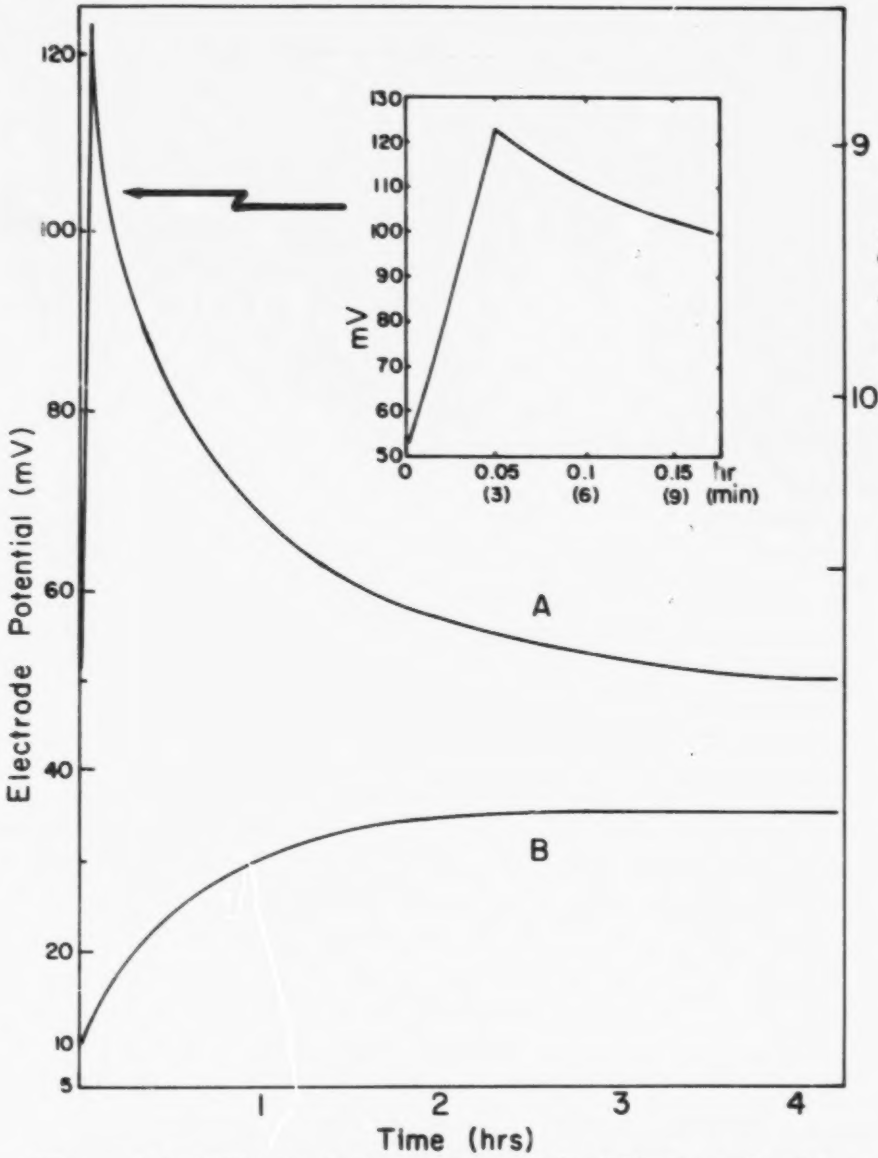

Figure 4-4. Effects of dissociation of Ca-EDTA on short term cupric ion activity after addition of $10^{-4.5}$M $Cu(NO_3)_2$.

Note: Background electrolyte 0.5 m KNO_3, pH 8.25 (2 x 10^{-3}M $NaHCO_3$ bubbled with air), $10^{-4.3}$M EDTA, temperature 23°C. A Radiometer selectrode (F 3000), an Orion d/j reference electrode, and an Orion pH electrode were used. In both experiments, the cupric ion activity (M) was calculated using the Nernst equation and data from 10^{-4}, 10^{-5} and 10^{-6}M $Cu(NO_3)_2$ solutions at pH 4:00 in 10^{-3}N KNO_3 background electrolyte. A) Calcium (10^{-2}M) in equilibrium with EDTA prior to copper addition; B) No calcium present.

EDTA in the presence of an excess of calcium: an initial peak in cupric ion activity is measured by the electrode, and it takes about four hours to approach the equilibrium value. Such phenomena have to be taken into account when studying the toxicity of metals to any aquatic organism, as transient effects can lead to large overestimations of toxicity.

The release of chelating metabolites has been widely assumed as a conditioning mechanism for culture media (39). As is the case for natural waters, most of the chemically quantitative work on this topic has focused on the synthesis and exudation of iron chelating agents, particularly hydroxamates (19, 30). What seems often overlooked is that hydroxamic acids do not chelate exclusively iron, and that their binding of other metals can result in sizable decrease of these metals' activities (1).

By direct potentiometric techniques, extracellular metabolites of algae have been characterized in terms of copper complexing capacity and affinity (48). According to this work, the ligand produced by the algae under the conditions of the experiments is characterized by a constant of approximately unity for the reaction:

$$Cu^{2+} + HY^{-} = H^{+} + CuY$$

If one assumes the ligand to be copper specific, the effect of its release in Aquil and Aquil with Tris is shown in Figure 4-5. Note that a significant decrease in the cupric ion activity does not begin until the total ligand concentration reaches 10^{-4}M, an upper limit for the measured ligand releases. Although $[Cu^{2+}]$ start decreasing at a slightly lower ligand concentration when the copper concentration is elevated, the release of such relatively weak complexing ligand has little overall effect on the cupric ion activity in a well chelated medium. Ligands, with higher affinity for copper, appear to be released by some blue green algae (22).

In principle, phytoplankton could modify the trace metal chemistry of the medium by assimilating artificial chelating agents. However, this potential problem is avoided by using EDTA or NTA which have been shown not to be assimilated by algae (23). Although photodegradation of EDTA and NTA has been reported (40), the light intensities normally used for culturing phytoplankton are insufficient to promote it in the laboratory.

PRECIPITATION

According to the computations of Table 4-1, the precipitation of several solids is calculated to be thermodynamically favorable in typical culturing media. Visible precipitates are indeed a common observation of users of algal

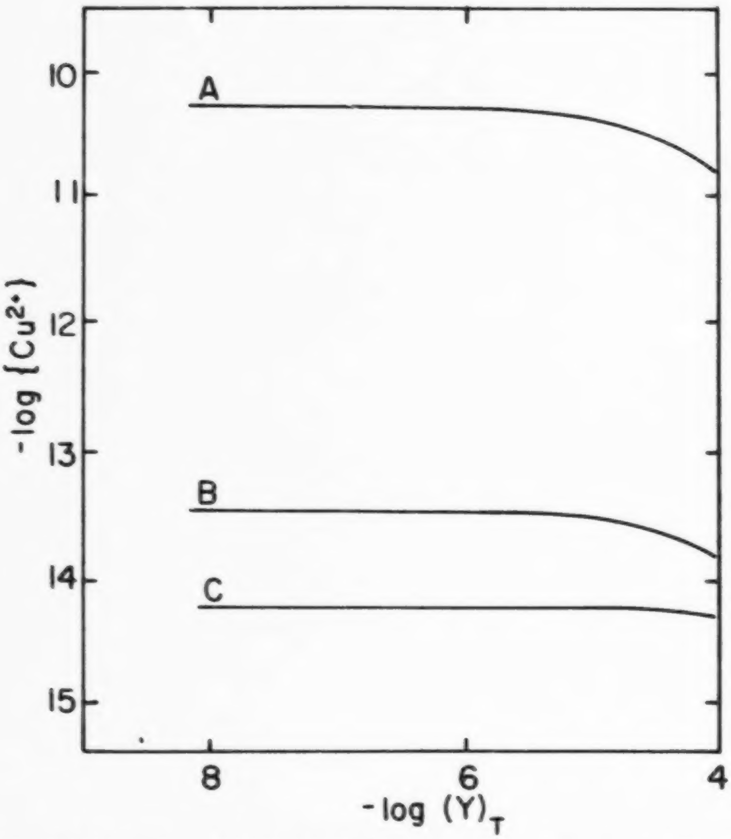

Figure 4-5. The effect of different molar concentrations of copper specific metabolite (Y) on the activity of the cupric ion for three variations of Aquil medium.

Note: A) $10^{-5.3}$M EDTA plus 10^{-6}M Cu; B) $10^{-6.3}$M EDTA plus 10^{-3}M Tris; C) $10^{-5.3}$M EDTA.

media. This is especially true following autoclaving, which brings about a large pH increase by eliminating carbon dioxide from the system. This problem has been studied by researchers involved in the design of culturing media (32, 8, 12, 18, 26). The increase in temperature and pH during autoclaving decreases the solubility of calcium carbonate, and results in the precipitation of a magnesium rich solid (this suggests the solid to be magnesium calcite, although aragonite has been identified in such precipitates). Hydrous oxides of iron and manganese can also precipitate under such conditions, depending on the chelating agent concentration and the pH reached during autoclaving. When such precipitates occur, phosphate becomes largely associated with the solid phase, presumably in some calcium precipitates (apatite or $CaHPO_4$), or as an adsorbate on the various solids. Depending on the initial concentration of silicic acid and on the nature of the container, which can increase the silicate concentration of the solution by dissolution, some amorphous or crystalline form of SiO_2 can form in the medium.

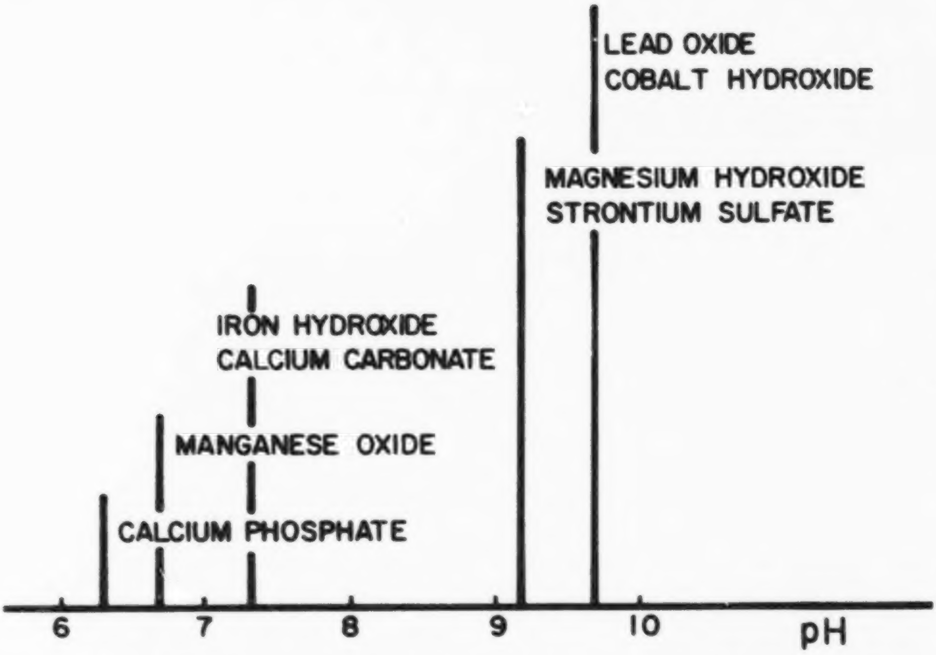

Figure 4.6. Saturation pH for various metal solids in Aquil.

These various solid formation processes are dependent on kinetic factors which are controlled by the particular temperature and pH regime of the medium. These, in turn, depend on the conditions and duration of autoclaving, as well as on the size of the containers and the mixing conditions. Precipitates are rarely seen with filter sterilization. Figure 4-6 shows the onset of saturation for various solids as pH is increased in the medium Aquil, normally designed to avoid precipitates. None of the four solids that are saturated at pH = 8 in Aquil are actually seen to precipitate, even after autoclaving if the volumes are kept smaller than 100 ml. If larger volumes are autoclaved, immediate bubbling with carbon dioxide prevents precipitation. Avoidance of calcium carbonate precipitate is very important for maintaining iron and manganese in solution, as the presence of $CaCO_3(s)$ will catalyze the formation of hydrous oxides of manganese and iron (43). For other trace metals the formation of these precipitates creates difficulties mostly through adsorption processes (see next section).

For toxicity studies, trace metals are sometimes introduced in algal cultures in excess of the chelating agent concentration. Precipitates are then often expected to form mostly oxides, hydroxides and carbonates, depending on the metal. For example, a hydroxide ($Cu(OH)_2$), an oxide (CuO, tenorite) and a carbonate ($Cu_2CO_3(OH)_2$, malachite) become quickly saturated in Aquil when copper exceeds the EDTA concentration. Although the hydroxide is not the thermodynamically stable form, it probably is the one which forms initially in the medium for kinetic reasons. Regardless of the precise nature of the solid, good agreement has been obtained between calculated and measured copper concentration in the solid phase in Aquil medium with a high EDTA concentration, 2 hours after addition of excess copper (29). It is worth noting that the precipitate was very finely dispersed, and that centrifugation was necessary to separate it from the aqueous phase. Ignorance of the formation of a precipitate can obscure completely the meaning of otherwise well controlled experiments. In terms of metal ion activity, the situation is complicated by the change in the nature of the precipitate which might evolve from an active form to a more stable one. In copper saturated media, the cupric ion activity has been measured potentiometrically to decrease markedly over 24 hours, the rate of decrease becoming very small thereafter (22). Such conditions can create large uncertainties in toxicity experiments.

ADSORPTION

The common notion that chelating agents make iron available to algae, seems to be supported by experiments where addition of iron or EDTA salts provide similar growth and carbon uptake enhancement in a variety of algal cultures (3). However, aluminium salts have also been observed to enhance carbon uptake (24). Following Stumm and Barber (41), it is now a prevalent interpretation of such experiments to attribute part, or all of the beneficial effect of the metal additions to a scavenging of other toxic metals by adsorption on precipitating iron or aluminium hydrous oxides. Figure 4-7 illustrates the beneficial effect of iron additions to a *Pyramimonas* culture, and demonstrates how iron and copper behave antagonistically under controlled conditions (28). The growth rate of *Pyramimonas* is reduced at a total copper concentration of 1.2 10^{-7}M, and completely stopped at 4.4 10^{-7}M when the iron concentration is low (1.2 10^{-6}M). Increasing the iron concentration by a factor of 10 completely blocks the toxic effect of the same copper concentrations. The question to be resolved is how much of this "detoxification" of copper by iron is due to adsorption processes, effectively removing the copper from solution and decreasing the cupric ion activity, and how much is due to a genuine physiological antagonistic effect at the cellular level. In a recent study of the adsorption of copper on hydrous iron oxide in seawater (48), it has been observed that under conditions similar to the experiments of Figure 4-7, iron adsorbs copper up to a Fe/Cu molar ratio of 1/3. Adsorption can then certainly account for all of the antagonistic effects in the *Pyramimonas* experiment. What becomes more difficult to explain is the lack of antagonistic effect at the low iron concentration (1.2 x 10^{-6}M) since even then the highest copper concentration (4.4 x 10^{-7}M) should be entirely adsorbed. Note, however, that this is a domain of concentrations where copper starts saturating the colloidal iron surface, and there must be a titration effect

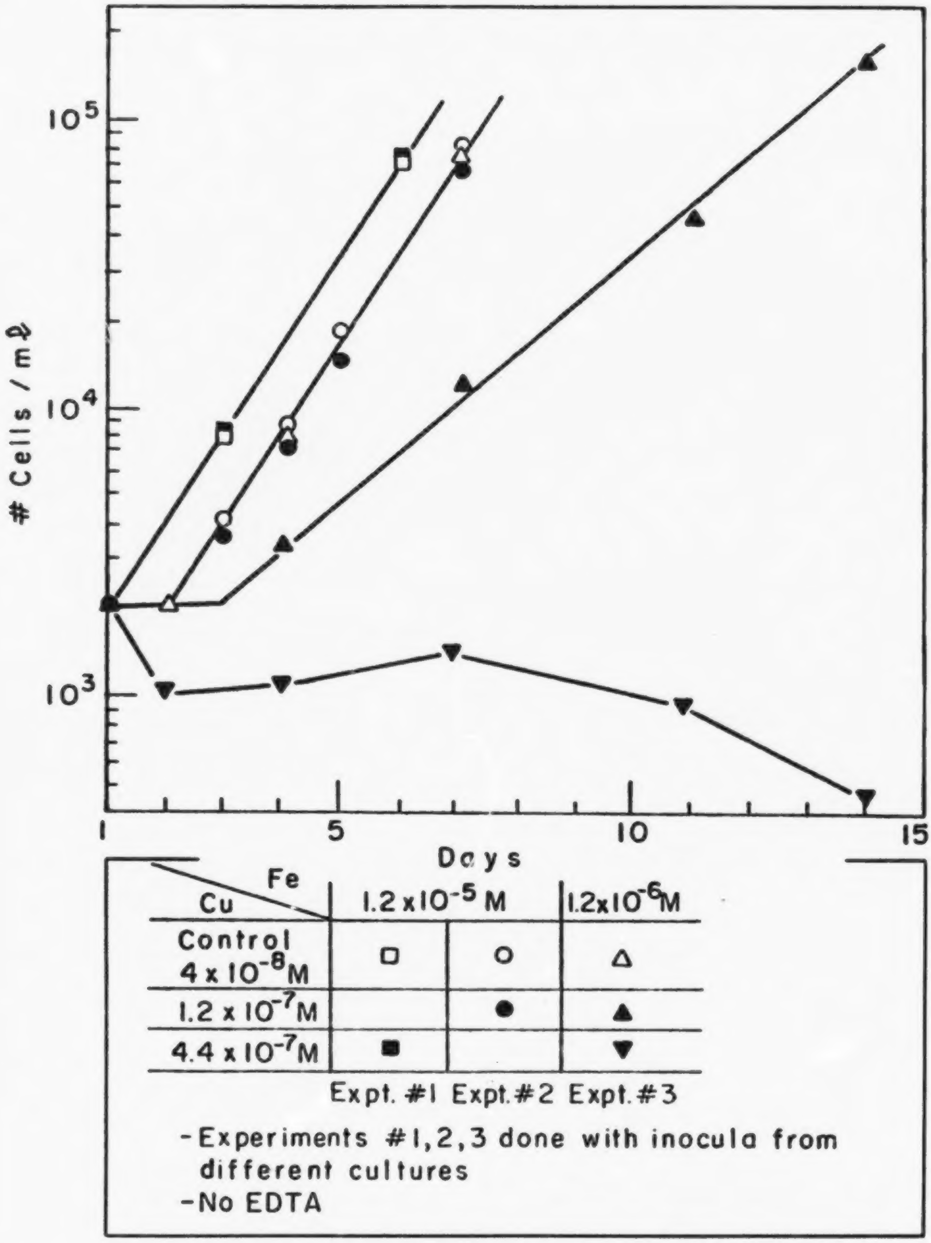

Figure 4-7. Antagonistic effects of iron and copper on the growth rate of Pyramimonas 1 in artificial seawater medium with the usual supplements of F/2 medium except for EDTA which is not added.

Note: Experiments 1, 2, and 3 represent inocula from different cultures. "Fresh" iron is $FeCl_3$ solution prepared the day of the experiment and sterilized by filtration.

(like that of copper on EDTA in Figure 4-3) where the cupric ion activity increases rapidly with increasing copper concentration. Exact quantitification of this phenomenon awaits a better mathematical description of adsorption processes on hydrous iron oxides in seawater. Despite great recent advances in the modeling of adsorption in aqueous systems (50, 42, 15), it is still the least quantifiable chemical process in thermodynamic calculations. The presence of precipitates in a culture medium modifies its global trace metal chemistry to an unpredictable degree. This creates the most common difficulty in interpreting experiments on toxicity of metals to a variety of organisms. Note that adsorption on the walls of a glass culture vessel is equally hard to predict. Choice of container material which minimizes adsorption of solutes is critical to the design of trace metal toxicity experiments.

Adsorption on the surface of algal cells can also be important for the trace metal chemistry of the medium in dense cultures. There is, however, no practical way to distinguish it from intracellular uptake. The effects of cellular uptake processes including adsorption on the cell surface, have been discussed earlier.

INDIRECT CHEMICAL EFFECTS

The general principles of coordination, precipitation, and adsorption which have been discussed heretofore, are readily understood and their importance in toxicity studies is usually recognized. What is less often perceived is the global interdependency of the chemistry of culture media, the indirect interactions (43, 27). For example, upon variations in the total copper concentrations, it is natural to relate the observed effects to changes in the cupric ion activity. However, as illustrated in Figure 4-8, activities of the zinc and ferric ions are also increased when the total copper is augmented in Aquil. Conceivably, any or all of these increased activities could be responsible for the observed effects. It is then a difficult choice to either maintain all metal activities constant by *adhoc* modification of all analytical concentrations—a method which multiplies the work for medium preparation and can create other interpretive ambiguities—or to perform the multitude of necessary controls on an already arduous series of experiments. Table 4-3 shows how the total metal concentrations have to be varied concomitantly with that of copper, to vary exclusively the cupric ion activity in Aquil with two EDTA concentrations (35).

The indirect interactions illustrated in Figure 4-8 are almost exclusively mediated by EDTA, which chelates all the interdependent metals. In principle, a convenient way to avoid the complications created by these interactions is to reduce them to a minimum. This can be achieved by uncoupling the system using more specific complexing agents. Figure 4-8 shows how the metal

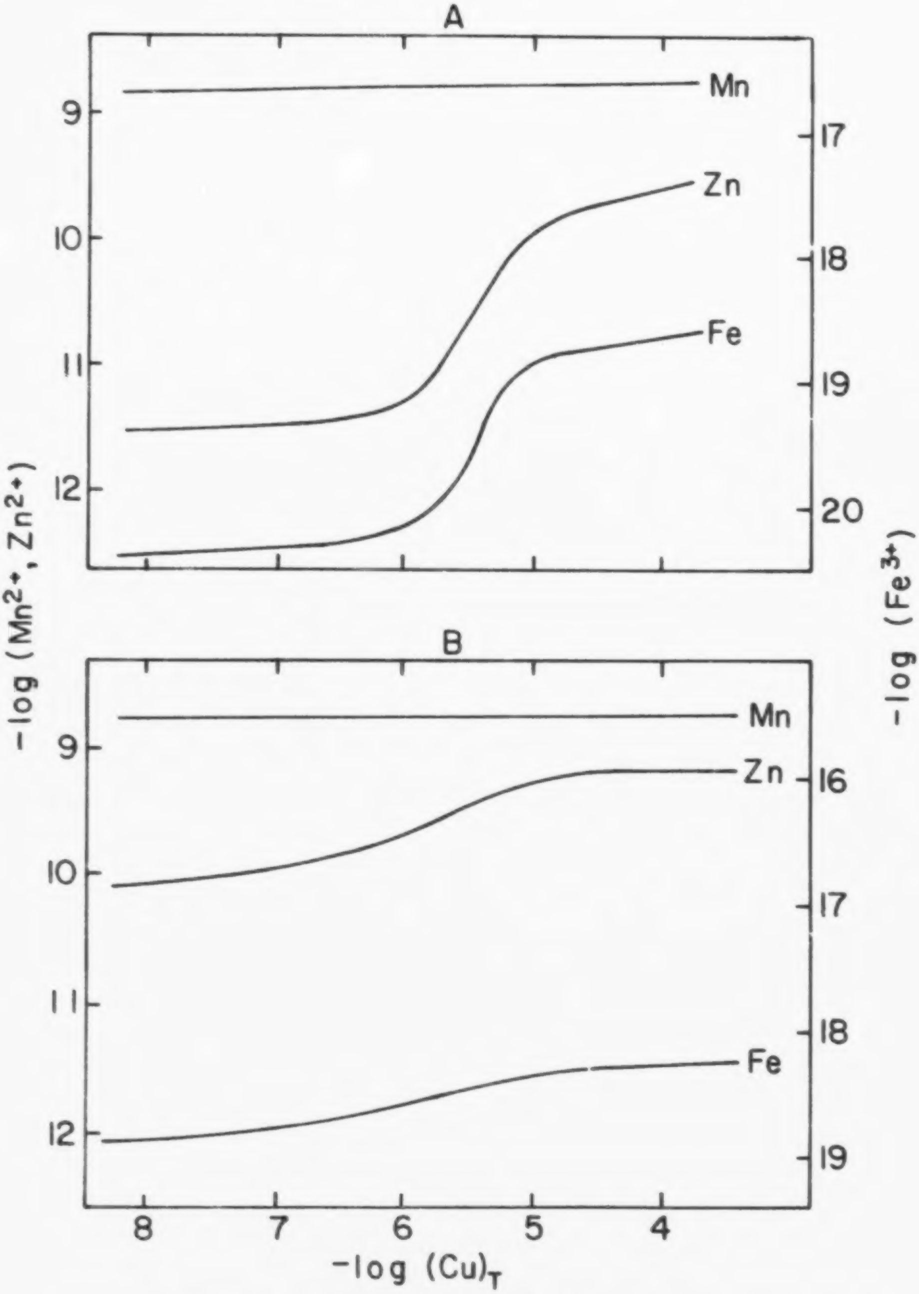

Figure 4-8. Variations in metal activities (M) of manganese, zinc and iron with total copper concentration.

Note: A) Aquil medium, $10^{-5.3}$M EDTA; B) Aquil with $10^{-6.3}$M EDTA, 10^{-3}M Tris.

Table 4-3. Calculation of Total Metal Concentrations Needed to Change the Cupric Ion Activity in Aquil While Maintaining the Other Metal Activities Constant (-log (concentration) or [activity], M)

$(\text{EDTA})_T$	$[\text{Cu}^{2+}]$	$(\text{Copper})_T$	$(\text{Iron})_T$	$(\text{Mang})_T$	$(\text{Zinc})_T$	$(\text{Cobalt})_T$
	8.5	3.30	7.00	7.63	8.7	8.6
3.3	10.9	3.70	4.72	6.40	6.49	6.7
	11.3	4.0	4.6	6.30	6.40	6.6
	9.8	4.35	6.45	7.20	8.30	8.49
4.3	10.9	4.70	5.72	7.20	7.49	7.7
	11.3	5.00	5.58	7.15	7.4	7.6

activities vary with total copper in modified Aquil medium containing "Tris", a ligand known to chelate mostly copper (44). Upon variations in copper concentration, the other metals are seen to have a much more constant activity in Aquil with Tris than in Aquil with only EDTA.

One of the principal ways by which indirect chemical effects can be initiated is through pH variations. For example, pH has an indirect effect on metal complexation due to the acid-base properties of the coordinating ligands. Figure 4-9 illustrates this effect for Mn, Cu and Zn in Aquil, with EDTA and Aquil with Tris. In this case, Tris mediates a much greater indirect effect than EDTA: zinc and especially cupric ion activities are markedly depressed by increasing pH in the Tris medium, while the activities of all three ions remain essentially constant in the EDTA medium. Increases in pH, which can be brought about by photosynthetic carbon uptake if the aeration of the culture is insufficient, can also result in precipitation as illustrated in Figure 4-6. Adsorption on the fresh precipitate will follow, resulting in an unquantified decrease in the soluble concentration of trace metals. It is apparent that pH is a major factor in determining directly and indirectly the activity and toxicity of trace metals, and should be monitored regularly in metal toxicity experiments.

CONCLUSION

The chemistry of metals in the external milieu of algal cells is only one of the determinants of their toxicity. The literature on bacteria and higher cells abounds with examples of how the sensitivity of a particular strain or clone to a particular toxicant, depends markedly on the physiological status of the cells (9, 4). Although it is often recognized that the same situation should apply to phytoplankton, this concept has received scant attention in recent algal literature. It stands to reason that the previous history of an algal cell, its

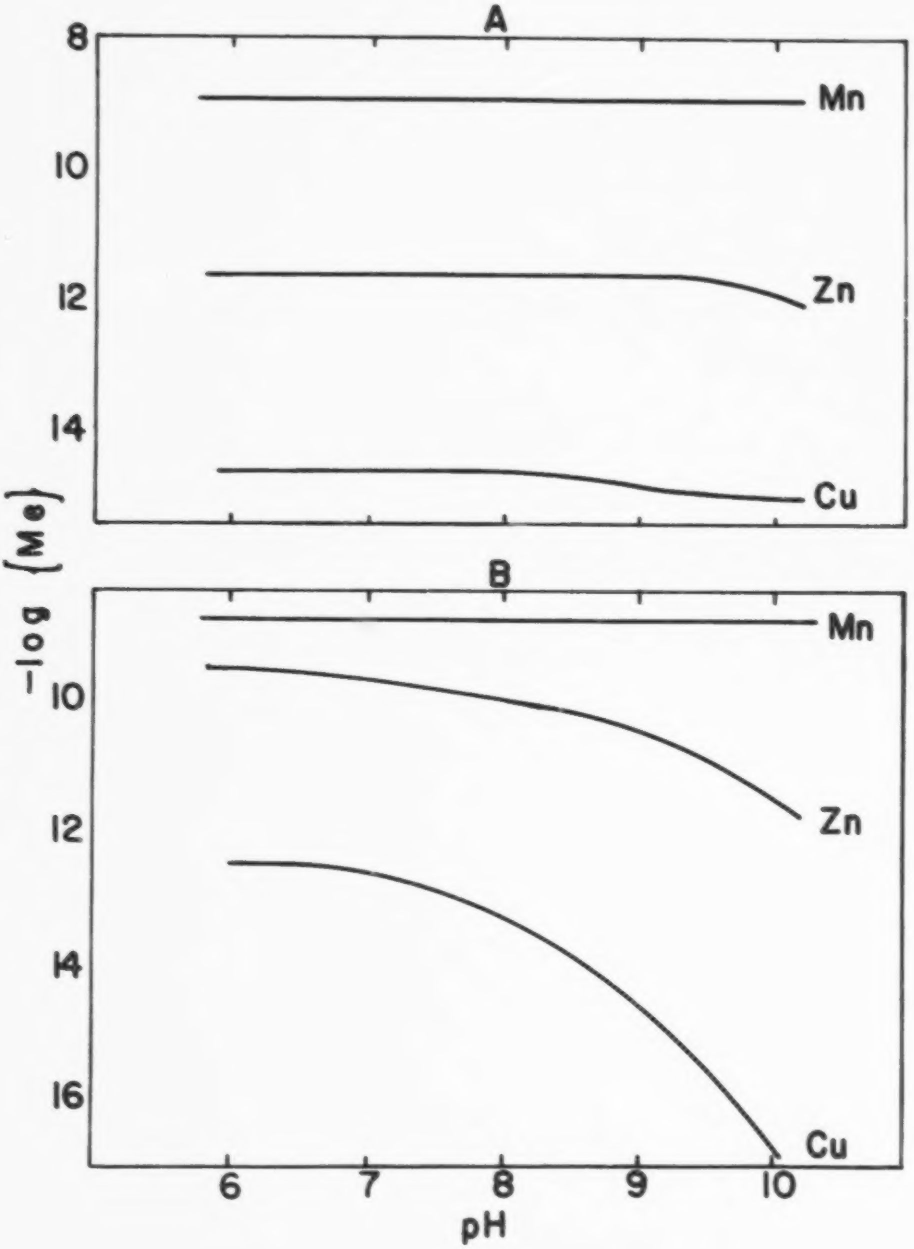

Figure 4-9. Variations in metal activities with pH.

Note: A) Aquil medium, $10^{-5.3}$M EDTA; B) Aquil with $10^{-6.3}$M EDTA, 10^{-3}M Tris.

nutritional status, and the particular phase of the cell cycle during which the experiment is conducted – to name but a few obvious determinants of physiological status – must affect its sensitivity to trace metals. Batch culture experiments which, so far, have been used principally for metal toxicity studies, have inherent restrictions to resolve the importance of these physiological factors. Toxicity studies in continuous phytoplankton cultures promise to be enlightening in this respect; they also promise to accentuate the difficulties in controlling precisely the chemistry of the system.

It is hoped that the conceptual framework presented here will help in designing and interpreting experiments where physiological responses to trace metal toxicity are clearly assessed, distinctly from purely chemical effects in the growth medium. It is also hoped that this study will help to increase phytoplankton physiologists' awareness of the important chemical processes which can affect their studies. It is, for example, surprising that so little attention has been paid to the possible importance of phosphate speciation in nutrient uptake experiments. Understanding the ecology of phytoplankton requires detailed resolution of the cells' physiological responses to the total aquatic chemistry of their environment.

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A SIMPLE ELUTION TECHNIQUE FOR THE ANALYSIS OF COPPER IN *NEANTHES ARENACEODENTATA*

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ABSTRACT

It is common practice to dissolve the tissue of marine organisms completely with acid prior to metal analysis with atomic absorption. However, it may not be necessary to completely destroy the organic matrix with acids prior to metal analysis. It has been determined that a simple 5 percent HNO_3 elution of a freeze-dried *Neanthes arenaceodentata* is sufficient to extract Cu quantitatively from this marine polychaete. This type of elution, rather than complete dissolution, has several advantages when analyzing small (1 mg to 10 mg) organisms. The two major advantages are (1) blank values are lower, and (2) the technique is less tedious and time consuming.

INTRODUCTION

High temperature ashing and/or various acids (HNO_3, H_2SO_4, $HClO_4$, etc.) are generally used to break down, oxidize, and solubilize marine organisms prior to metal analysis by atomic absorption. If metal levels are high, and the organisms weigh several grams, the techniques of dry ashing and wet ashing are usually successful. However, solubilizing individual organisms that weigh 1 mg to 10 mg with standard techniques without contaminating the final solutions for the element of interest is difficult.

Matsunaga (1) has shown that it was possible to extract Hg completely from various types of fish muscle with 1N HCl containing cupric chloride. In his study, no attempt was made to solubilize the fish muscle tissue. Therefore, we reasoned that a simple elution with 5 percent HNO_3 might be sufficient to extract metals from small marine organisms. The feasibility of extracting Cu by this elution technique was tested on the polychaete, *Neanthes arenaceodentata*.

EXPERIMENTAL METHODS

Apparatus

All atomic absorption analyses were made using a Perkin-Elmer atomic absorption unit (Model 360) coupled to a Perkin-Elmer heated graphite atomizer (Model No. HGA-2100). The weight measurements of the worms were made with a Perkin-Elmer microbalance (Auto balance Model No. AD-2Z). Freeze-drying of the worms was accomplished using a Virtis (Model No. 10-145MR-BA) lyophilizer. Low temperature ashing of the samples was done with a L.F.E. (Model LTA-505) low temperature asher.

Reagents and Materials

Ultrex HNO_3 was used throughout the analytical elution and dissolution procedures. Copper standards were made up from a stock solution of ALPHA atomic absorption standard copper. All 2/5 dram polyethylene snap-cap vials were acid washed in concentrated HNO_3 for two days, soaked in demineralized water for two days, and finally rinsed five times with copious quantities of demineralized water. The vials were allowed to air dry in a class-100 clean bench.

Procedure

The 61 polychaete specimens used in this study were raised in the laboratory. Complete details of the methods to raise the worms are given by Pesch and Morgan (2). Live polychaete samples were removed from the seawater tanks with the aid of a nylon brush and rinsed in control seawater for approximately one minute, and then placed in precleaned polyethylene vials (1.2 ml capacity) fitted with snap-caps. The samples were frozen and then freeze-dried for 24 hours. The freeze-dried worms were then weighed. The average weight was 8.7 ± 4.7 mg. One ml of 5 percent HNO_3 was added to the worms in their respective vials. The sample vials were capped and allowed to stand at room temperature for two days. The worms were then transferred from the first extraction vial (A) to precleaned tared vials (B) with the aid of a teflon fiber. The tared vials (B) containing the wet acid leached worms were again weighed. One ml of 5 percent HNO_3 was added to the B vials. The (B) vials were capped and allowed to stand at room temperature for two days. The worms were again transferred to pretared vials (C) and weighed wet. The worms were then freeze-dried and weighed again. During these transfer steps care was taken so that the worms did not disintegrate. The insoluble worm carcasses were then destroyed by low temperature ashing. The freeze-dried worms were inserted into teflon beakers (10 ml capacity) and ashed for 24 hours at the following conditions: O_2 flow 50 cc/min; and RF power of 50 watts. After ashing, the inorganic residue was transferred back into the (C) vials. The transfer was facilitated by adding 0.1 ml of ultra-pure concentrated HNO_3 to the teflon beaker and slowly picking up the inorganic residue into the drop of HNO_3 as it was rolled around the inside of the beaker. The HNO_3 does not wet the inside of the beaker and can be quantitatively transferred into the polyvial. The (C) vials were capped and allowed to stand at room temperature for several days to insure dissolution of the particulate residue. One ml of demineralized water was added to the (C) vials after the dissolution period. The final acid concentration in the (C) vials was approximately 1.6 N in HNO_3. All three vial solutions (A, B and C) were then analyzed for their Cu content.

RESULTS AND DISCUSSION

Three of the (A) vial solutions were monitored for increases in Cu content during the first 15 hours of the extraction process. This data is plotted in Figure 5-1. It can be seen from Figure 5-1 that the extraction appears to be fairly rapid, and approaches a constant value at 15 hours. This particular data was the major reason for selecting a two-day elution time for the rest of the worms processed.

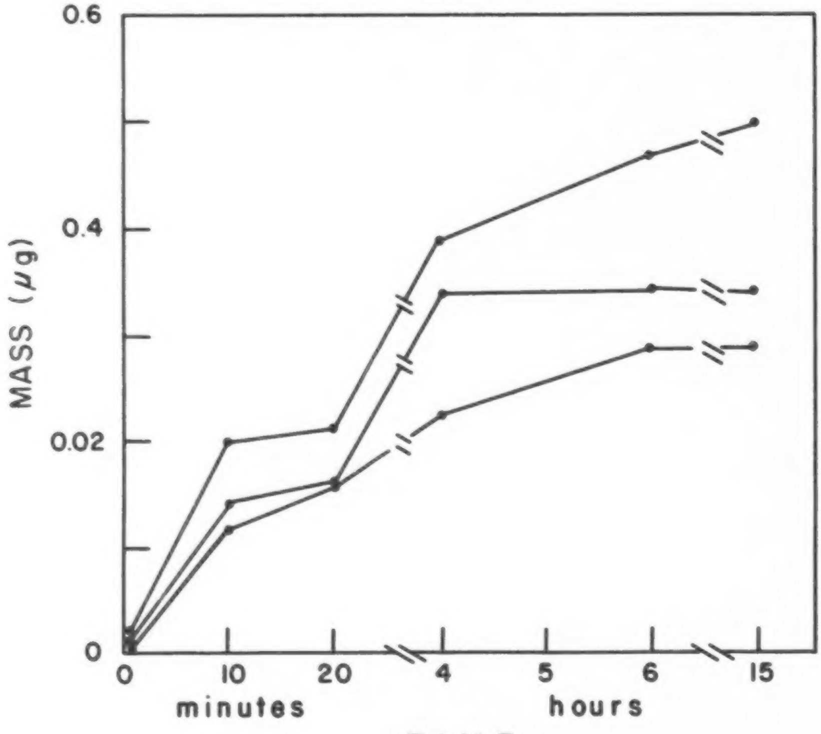

Figure 5-1. Extraction of Cu from Neanthes arenaceodentata with 5 percent HNO_3 as function of time.

The weight measurements made during the various processing steps allowed the calculation of the amount of 5 percent HNO_3 transferred with each worm from vial (A) to vial (B), and from vial (B) to vial (C). Therefore, the amount of solubilized Cu transferred during the transfer steps could be calculated. The amount of 5 percent HNO_3 transferred from the (A) vial to the (B) vials ranged between 7 and 20 percent of the 5 percent HNO_3 present in the (A) vials. Figure 5-2 shows the calculated mass of Cu that should be present in the (B) vials versus the measured mass of Cu present in the (B) vials. The solid line represents a perfect correlation, and is not the calculated regression line. This plot shows that there is very little copper that cannot be accounted for in the second elution that has not been transferred from the first solution.

Figure 5-3 shows a plot of the original freeze-dried weights of the worms, versus the freeze-dried weights of the worms after two elutions in 5 percent HNO_3. It is interesting to note that the worms lost 50.5 ± 7.2 percent of their weight with the two elutions. The Na concentrations were measured in all samples, and indicated that only about half of the freeze-dried weight loss could be attributed to the loss of solubilized NaCl. Most of the unexplained weight loss probably comes from solubilized organic matter. On a qualitative basis, this was confirmed by the color of the (A) and (B) solutions which were pale yellow in color, and contained very surface active compounds. Even though the worms may have been slowly dissolving in the 5 percent HNO_3, only a few of the worms had broken down into two or more pieces, and generally from a physical appearance looked unchanged.

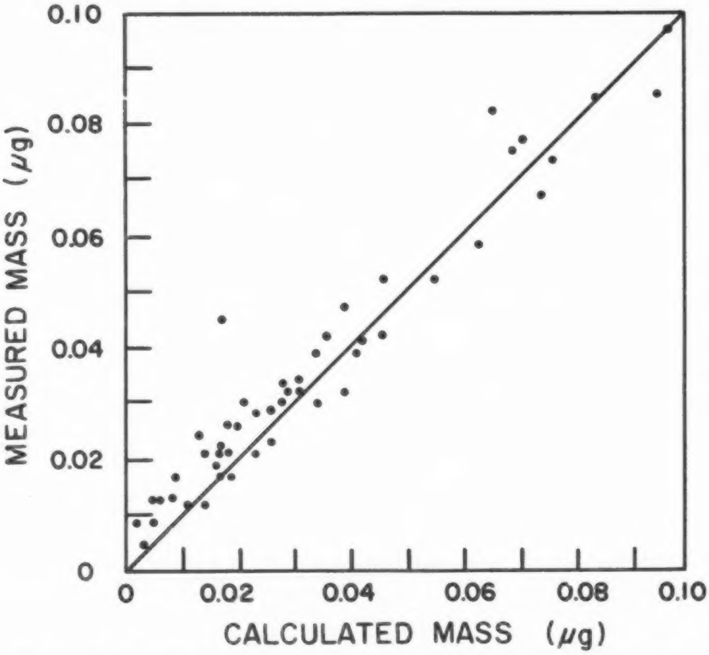

Figure 5-2. Calculated mass of Cu in the (B) vials versus the measured mass of Cu in the (B) vials.

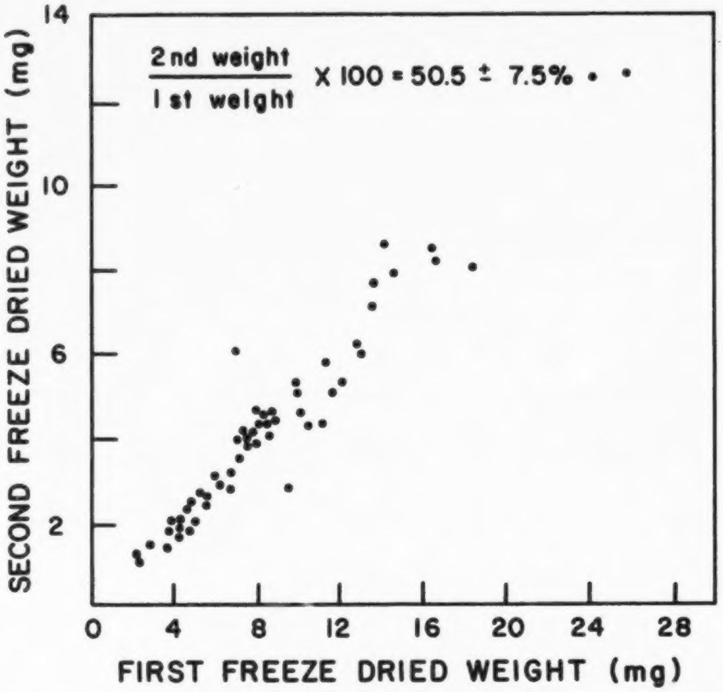

Figure 5-3. First freeze-dried weight versus the second freeze-dried weight of Neanthes arenaceodentata samples.

The elution efficiency (EE) of the first extraction, versus the total Cu present in the worms, was calculated by the following equations:

$$EE\% = \frac{C_{1M}}{C_T} \times 100$$

where $C_T = C_{1M} + (C_{2M} - C_{2C}) + (C_{3M} - C_{3C})$

and C_T = Total mass of Cu
C_{1M} = Measured Cu mass in the (A) vial
C_{2M} = Measured Cu mass in the (B) vial
C_{3M} = Measured Cu mass in the (C) vial
C_{2C} = Calculated Cu mass in the (B) vial
C_{3C} = Calculated Cu mass in the (C) vial

The calculated EE% of Cu for this set of samples was 97.8 ± 1.8 percent. Therefore, for all practical purposes of analysis for environmental samples, the first extraction of the worms with 5 percent HNO_3 is essentially complete for Cu.

Using this elution technique it is possible to extract and analyze large numbers of worms for copper very simply, since only the first extract need be analyzed. The methods and conditions used in this study should not be considered to be the ultimate in elution of metals from marine organisms. It may be possible to use other dilute acids (i.e. HF, HCl, H_2SO_4, etc.) that may be more effective for the elution of other metals from different species. If elution rather than total destruction of the animal matrix is desirable, then a thorough study should be made of the effectiveness of the procedure chosen. Dilute acid elution has several advantages over complete destruction of the sample matrix. The first and most important is the potential for providing lower blanks. The second is the simplicity involved, which allows processing 100 small organisms in approximately 8 contact hours. The worms need not be removed from their respective extraction vials prior to analysis, since they sink to the bottom of the vial and do not interfere with HGA atomic absorption analysis. The samples do not need to be analyzed within any constrained time frame. Some samples have been analyzed repeatedly over a period of several months, and have shown no tendency for a concentration change with respect to Cu. The worms also do not decompose over this period of time, as they appear to be permanently preserved. At this time, we have used this elution technique to analyze over 1000 small marine organisms for Cu.

ACKNOWLEDGMENTS

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GEOCHEMISTRY OF FOSSIL FUEL HYDROCARBONS IN MARINE SEDIMENTS: SELECTED ASPECTS

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ABSTRACT

Three investigations are described which illustrate recent advances in analytical chemical and geochemical research on fossil fuel hydrocarbons in the marine environment.

First: The application of quantitative gas chromatography-mass fragmentography to measure selected aromatic hydrocarbons in marsh and coastal sediments. Instrument precisions of 2 to 3% for 50 x 10^{-9} g of naphthalene and 1-methylnaphthalene are achieved. The detection limit for naphthalene (signal/noise ratio of 2:1) is estimated to be 5 x 10^{-11} g/g dry weight of sediment with 25-50 g dry weight silt-clay coastal sediments. Using this method No. 2 fuel oil aromatic hydrocarbons incorporated into marsh sediments were precisely measured in samples taken within one week of a spill, and eight months after a spill.

Second: Several sections from a core in Buzzards Bay, Massachusetts have been analyzed for alkanes, cycloalkanes, and aromatic hydrocarbons. This is an initial attempt at investigating an historical record of anthropogenic fossil fuel inputs to coastal sediments. The results indicate an increase of an order of magnitude in concentrations of fossil fuel hydrocarbons from circa 1810 to 1840 to the present. The aromatic hydrocarbon distributions indicate urban air hydrocarbons as the major source.

Third: The input of fossil fuel hydrocarbons from sewage sludge and dredge spoils in the New York Bight is discussed. An estimated 3.6 x 10^3 tons of fossil fuel hydrocarbons are discharged each year by dumping in this area.

INTRODUCTION

Research concerned with chemical pollutants in the environment can be most easily divided into two broad areas of investigation: biological effects and biogeochemistry. The latter is the category encompassing the three fossil fuel pollution investigations summarized in this paper. Each investigation is, or will be, the subject of one or more papers, and the reader is referred to these papers for details and further discussion. Biogeochemical research delves into the sources, distributions, pathways of transfer, reactions, intermittent and ultimate fates of pollutants in the environment.

The incorporation of fossil fuel hydrocarbons into surface sediments as a result of oil spills or chronic effluent releases (2, 6, 15), and the resulting long-term slow (years) chemical and biochemical removal processes, was a major finding of oil pollution research between 1969 and 1974. An important concern evolving from these findings was the question of the distribution and long-term fate of fossil fuel hydrocarbons in surface sediments. The fossil fuel components causing the greatest concern were the aromatic hydrocarbons, although recent research has documented that nitrogen containing compounds such as p-toluidines and degradation products such as phenalene-1-one, are also very toxic to certain marine species (18, 19). Thus, there was a need for investigations of aromatic hydrocarbons in surface sediments. This led to a search for a means to accurately measure individual aromatic hydrocarbons at the 1 to 100 x 10^{-9} g/g dry weight concentration level in sediments. Quantitative gas chromatography-mass spectrometry or mass fragmentography has evolved as one of the more discriminating and sensitive methods to apply to this problem (10, 11, 13).

Our quantitative GC-mass fragmentographic method is described in detail in another paper (8). We have determined the precision of the method as 2 to 3% based on repeated injections of standard aromatic hydrocarbons for 50 x 10^{-9} g and about 12% for 1 x 10^{-9} g. This compares favorably with quantitative gas chromatographic determinations. However, the GC-MF technique has the very powerful added advantage of allowing mass spectrum to be scanned to insure more complete identification of the compounds measured. A comparison of GC-MF determination of the weight percent of selected aromatic hydrocarbons in the API reference No. 2 fuel oil with earlier GC measurements (17) is presented in Table 6-1. We think that the agreement is quite good. We have applied this technique to measuring selected aromatic hydrocarbons in marsh sediments exposed to a No. 2 fuel oil spill.

RESULTS AND DISCUSSION

Bouchard No. 65 Oil Spill—October, 1974

On October 12, 1975 the Bouchard Barge No. 65 spilled No. 2 fuel oil into Buzzards Bay, Massachusetts. A small amount of this oil entered Windsor Cove

Table 6-1. Weight Percent of Selected Aromatic Hydrocarbons in Fuel Oils.

	Naphthalene	C_1-Naphthalenes	C_1-Phenanthrenes	C_2-Phenanthrenes
API No. 2 Fuel Oil (Present Study)	–	2.0 ± 0.3	0.24 ± .02	0.23 ± .03
API No. 2 Fuel Oil (Warner, Ref. 17)	0.40	2.7	0.27	0.19
Bouchard Barge No. 65 No. 2 Fuel Oil Spilled October, 1974. Buzzards Bay, Mass.	0.17 ± .01	0.95 ± .05	0.37 ± .02	0.33 ± .04

(Figure 6-1) and a sheen of oil with accompanying fuel oil odor was present in some marsh and intertidal areas of the cove. We selected two sites for a small study of the long-term fate of this fuel oil in sediments; a marsh area and an intertidal area. The locations of these sites were carefully recorded, and cores have been taken every fall in October, and every spring in May or June since October, 1974. We did not intend, nor do we pretend, to offer an in-depth study of the geographical extent of the spill or long-term fate at several stations as was conducted for the West Falmouth oil spill (2, 3, 5). Funding, manpower, laboratory space and other commitments to oil pollution research prevented such a study. Also, it was our understanding that Commonwealth of Massachusetts laboratories were conducting a survey of the geographical extent, and long-term fate of the oil.

Our intent was to compare the long-term fate of the oil at the two locations described, with earlier studies of the West Falmouth oil spill. In essence, there was a near duplicate experiment in progress. The West Falmouth oil spill involved No. 2 fuel oil spilled in late September, 1969 a few miles away from where the Bouchard Barge No. 65 spilled No. 2 fuel oil in October, 1974 (Figure 6-1). Was the West Falmouth oil spill really unique with respect to longevity of the spilled oil in marsh and intertidal sediments as some have suggested? This was the primary focus of our investigation. The complete set of data of our study will be presented elsewhere. We have applied the GC-MF technique to a set of marsh cores from October, 1974 and May, 1975. This data is presented in Table 6-2. Note that the concentrations of aromatic hydrocarbons in the 14-18 or 15-20 cm core section are the concentrations present in marsh sediments prior to the spill. The concentrations of aromatic hydrocarbons in the surface sediments, 0-6 cm and 0-5 cm, clearly show at least two orders of magnitude elevation in concentration as a result of the fuel oil spill; and elevated concentrations are still present in May, 1975, although they have decreased by a factor of about 5 to 6. The longevity of the aromatic hydrocarbons in the marsh sediment is still under investigation.

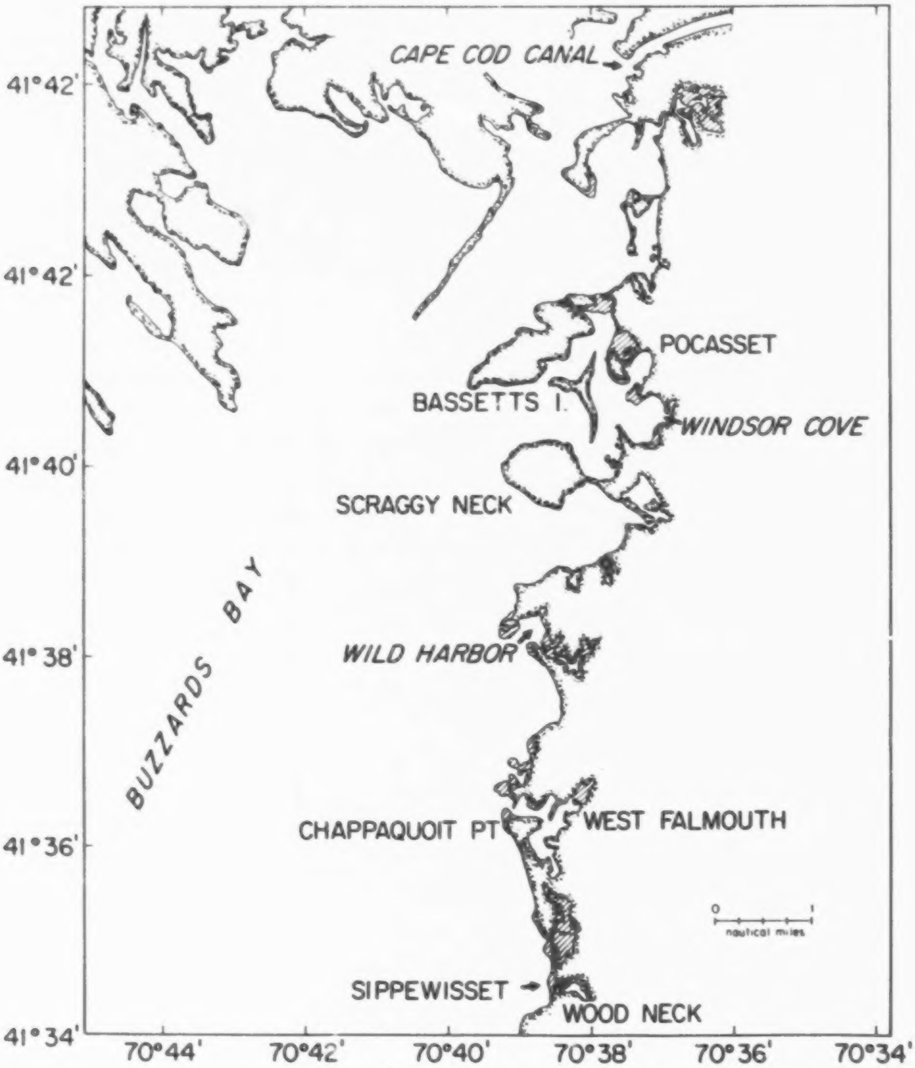
CAPE COD CANAL
POCASSET
BASSETTS I.
WINDSOR COVE
SCRAGGY NECK
BUZZARDS BAY
WILD HARBOR
CHAPPAQUOIT PT
WEST FALMOUTH
SIPPEWISSET
WOOD NECK
0
1
nautical miles
41°42'
41°42'
41°40'
41°38'
41°36'
41°34'
70°44'
70°42'
70°40'
70°38'
70°36'
70°34'

Figure 6-1. Buzzards Bay, Massachusetts.

Table 6-2. Concentrations of Naphthalenes/Phenanthrenes in Windsor Cove Sediments (μg/gram dry wt. sediment).

	MARSH			
	Oct.		May	
Component	Depth	Conc.	Depth	Conc.
Naphthalene	0-6 cm	9.2	0-5 cm	0.63
	14-18 cm	0.024	15-20 cm	0.011
C_1-Naphthalenes	0-6 cm	370	0-5 cm	41
	14-18 cm	1.1	15-20 cm	0.33
C_2-Naphthalenes	0-6 cm	1380	0-5 cm	260
	14-18 cm	5.1	15-20 cm	1.5
C_3-Naphthalenes	0-6 cm	3040	0-5 cm	590
	14-18 cm	12	15-20 cm	3.4
C_1-Phenanthrenes	0-6 cm	500	0-5 cm	97
	14-18 cm	2.2	15-20 cm	0.94
C_2-Phenanthrenes	0-6 cm	480	0-5 cm	96
	14-18 cm	2.2	15-20 cm	1.1

The data in Table 6-2 demonstrate the usefulness of the technique of quantitative GC-MF analysis in biogeochemical studies of oil spills. The data also demonstrates that fuel oil aromatic hydrocarbons have survived for at least seven months in the marsh sediments in concentrations well above background. Thus, for this time period, the West Falmouth spill was not unique. Further investigation will determine if the parallel between the fate of the petroleum compounds from the two oil spills will continue.

Historical Record of Fossil Fuel Hydrocarbons in Buzzards Bay, Massachusetts

Our gas chromatographic measurements of hydrocarbons in surface sediments at several locations in Buzzards Bay, Massachusetts indicated the presence of an unresolved complex mixture of hydrocarbons with a wide molecular weight range, indicating that these hydrocarbons might be from chronic oil pollution (8). However, there were several other sources such as natural diagenetic processes and weathering of ancient sediments to be considered (8). In order to assist in evaluating the source of these hydrocarbons, we obtained sediment cores at several locations in coastal areas of the western North Atlantic (8). At Station P Pb-210, geochronology measurements were obtained (7). These measurements and the measurements of Pu-239/240 and Cs-137 at this same location by others (4) allowed us to estimate sedimentation rates. We then measured hydrocarbon concentrations in several core sections at Station P. We also applied quantitative GC-MF analyses to measure phenanthrene and C_1 and C_2 phenanthrenes. The results of these measurements, as reported in (8), are given in Table 6-3. It is clear that circa 1900 concentrations of hydrocarbons constituting the unresolved complex mixture increased, as did the concentrations of phenanthrenes. The ratios of the C_1 and C_2 phenanthrenes are not those found in spilled oil. Instead, the ratios indicate that these aromatic hydrocarbons are from pyrolytic sources (13, 20). Our hypothesis is that these hydrocarbons are primarily from direct and remobilized urban air hydrocarbons (8). We have determined that there is a trend of decreasing concentrations of UCM hydrocarbons with increasing depth in a core at another station in Buzzards Bay (8), and a station in the Gulf of Maine (Figure 6-2).

Furthermore, similar results have been reported for Lake Washington, Seattle, Washington sediments (16). A much more detailed analysis of the aromatic hydrocarbons in three sections of another core from Station P,

Table 6-3. Hydrocarbons and Chlorinated Hydrocarbons in Station P Core Sections

Section	Average Time of Deposition	UCM[a] (μg/g)	Phenanthrenes (ng/g)		
			C_0	C_1	C_2
0-1 cm		74	NA[b]	44	38
1-2 cm		105	34	27	28
8-12 cm	1940	44	NA	31	29
20-24 cm	1900	12	15	11	10
54-58 cm	1790	5.2	3.7	2.9	2.8
58-62 cm	1780	6.2	NA	NA	NA

[a] Mixture of alkanes and cycloalkanes — indicates petroleum hydrocarbons.

[b] NA — not analyzed.

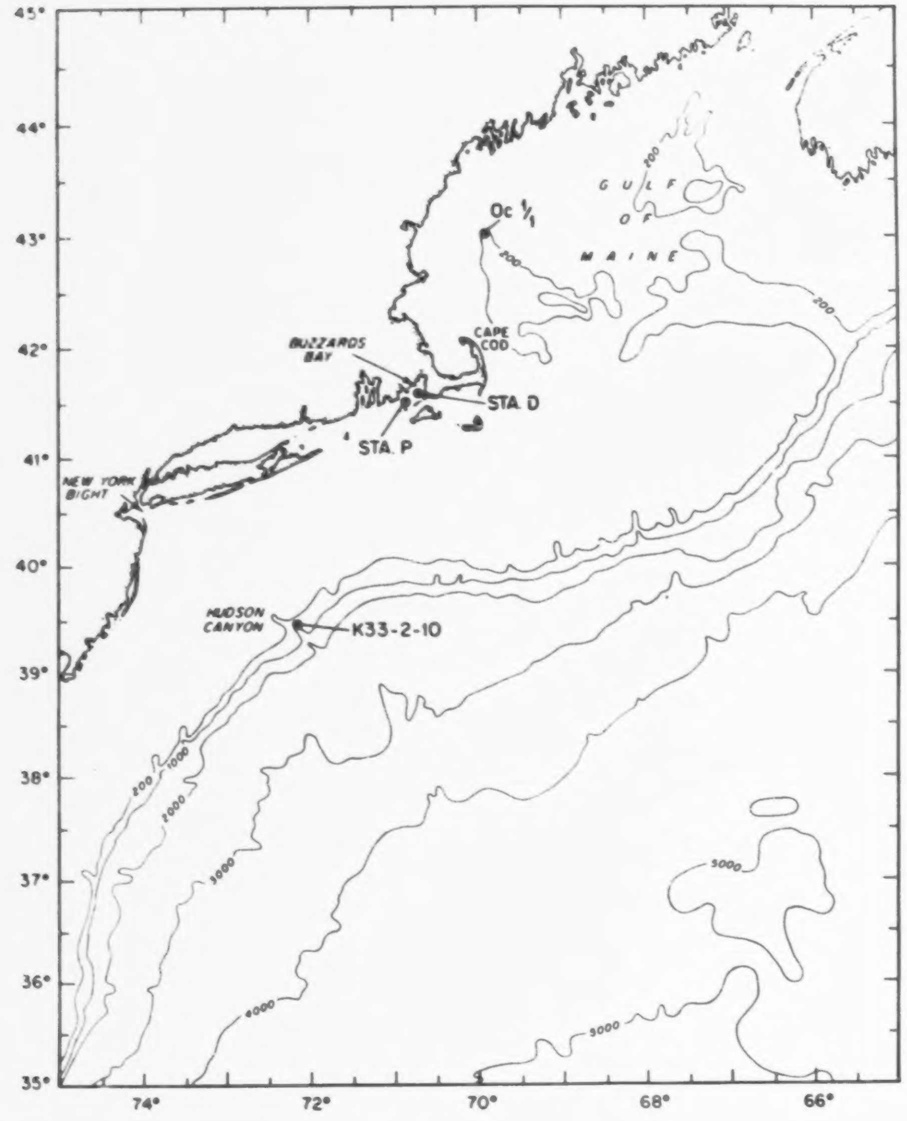
45°
44°
43°
42°
41°
40°
39°
38°
37°
36°
35°
74°
72°
70°
68°
66°
GULF OF MAINE
Oc 1/1
200
CAPE COD
BUZZARDS BAY
STA. D
STA. P
NEW YORK BIGHT
HUDSON CANYON
K33-2-10
1000
2000
3000
4000
5000

Figure 6-2. Gulf of Maine.

Buzzards Bay, have recently been completed (12). These analyses, also by GC-MF, greatly extend the earlier analyses for the Buzzards Bay station, and establish that polynuclear aromatic hydrocarbons (PAH) from a combustion source increase by at least an order of magnitude in sediment deposited after about 1850. Another recent paper (14), has reported a detailed study of PAH in a core from Lake Constance in the Federal Republic of Germany. The surface sediments of this core contained increases of PAH concentration of 50 to 100 times that of sediments deposited circa 1800. The PAH composition again indicated a pyrolytic source.

The implications of these findings are that coastal and lacustrine environments, especially the benthic ecosystems, have been exposed to increased PAH concentrations over the past several decades. Whether or not this chronic long-term increase in PAH concentration reflects a substantial environmental risk is not known, and a detailed discussion is beyond the scope of this paper. It is important, though, to consider that many of the PAHs are known to have adverse effects on marine organisms (13). The benthic ecosystems may have been "stressed" by PAH pollution for some time. This is an important point to keep in mind when considering control stations for studying oil spills in coastal areas. For example, the control stations for the studies of the effects of the West Falmouth oil spill on subtidal benthos were not very far from the two stations, P and D, we have sampled in Buzzards Bay. Does this mean that these stations are truly "normal" with respect to the effects of aromatic hydrocarbons, or have they also been subtly, chronically affected by the increasing amounts of PAH deposited from direct and remobilized urban air PAH?

New York Bight Surface Sediments

We have estimated the rate of fossil fuel hydrocarbons discharged by dumping in the New York Bight is about 3.6 x 10^3 tons/year (9), or about 2% of the estimated global discharge of 180 x 10^3 tons per year of petroleum hydrocarbons from routine operations and spills associated with outer continental shelf drilling and production (3). The composition of PAH in the New York Bight dump site surface sediments indicates that these hydrocarbons are primarily of pyrolytic origin (13). The fossil fuel hydrocarbons most likely are from urban air fallout, and are swept into storm sewers and municipal sewers by rain water, and are either discharged to New York harbor or become associated with the sewage sludge in the treatment plants. Dredge spoils from the harbor and sewage sludge are then dumped in the New York Bight resulting in delivery of PAH and other pollutants to the continental shelf area. Since there are other dump sites off the East coast of the U.S., the input of petroleum hydrocarbons from this source must be larger than in the New York Bight alone. Thus, significant and measurable quantities of contaminant hydrocarbons are already being deposited in continental shelf areas off the eastern United States before Outer Continental Shelf oil and gas drilling and production have begun. This must be taken into account when assessing potential environmental impacts of OCS operations, now and in the future.

SUMMARY AND GENERAL DISCUSSION

Aromatic hydrocarbons are incorporated into surface sediments as a result of oil spills, and the chronic dribbling of urban air hydrocarbons into the marine environment. These compounds are known to have adverse effects on marine organisms under certain conditions. The challenge posed is to conduct experiments which will investigate how bottom current resuspension, bioturbation by animals, and long-term microbial and chemical processes act individually and collectively on the aromatic hydrocarbons in surface sediments. Are these compounds in the sediments incorporated into benthic organisms? At what rate and under what conditions? We need to relate chemical analyses by some means to biological availability.

Some recent investigations conducted on a short-term two-week exposure of sipunculid worms suggest that naphthalenes can be ingested from naphthalene contaminated sediments (1). Two weeks of "depuration" in a clean environment removed all measurable quantities of naphthalenes from the worms (1). The exposure time was very short. What happens when exposure of the benthic organism is continuous for years, as is probably the case for low concentrations of polynuclear aromatic hydrocarbons in Buzzards Bay, and higher concentrations near the New York Bight area?

ACKNOWLEDGMENTS

I wish to thank R.A. Hites, R.E. LaFlamme, B.W. Tripp, N.M. Frew and J.M. Teal for enjoyable collaboration and discussion of much of the research discussed and referenced in this paper. This paper presents a synopsis of several papers and acknowledgments to agencies providing financial support are found in the referenced papers.

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IDENTIFICATION OF ENVIRONMENTAL GENETIC TOXICANTS WITH CULTURED MAMMALIAN CELLS

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ABSTRACT

Experiments designed to detect small-scale mutations leading to auxotrophy were carried out *in vitro* with the Chinese hamster ovarian (CHO) cell system (5-bromodeoxyuridine/visible light selection) initially described by Puck and Kao (43). The system was standardized with ethylmethanesulfonate (EMS), a known mutagen previously demonstrated to be active in CHO cells (27), and 5-bromodeoxyuridine (BrdU), another known mutagen (7) utilized in the selection procedure, but not previously evaluated for mutagenic activity in the CHO Cell/BrdU-VL assay. Both EMS and BrdU routinely yielded glycine, hypoxanthine or triple-requiring (glycine/hypoxanthine/thymidine) auxotrophs and showed dose response. For a series of inorganic compounds known to be or suspected of being genetic toxicants, statistically significant numbers of auxotrophs were obtained only with the chloride salts of cadmium and manganese. Neither cadmium nor maganese were consistently mutagenic, cadmium showing activity in about 20 percent of experiments, manganese in 50 percent of experiments. It was not possible to demonstrate dose response with these compounds. A water extract of JP-5 jet fuel was also found to be mutagenic in a single test. Variant cell types other than auxotrophs were isolated from cell populations treated with three different carcinogenic agents (EMS, CrO_3, $PbAc_2.3H_2O$) but not from control experiments. These cells, exhibiting either a rounded cell morphology or potential contact inhibition, may reflect mutation in additional loci of possible value as genetic markers. Other data are presented to illustrate special problems associated with the application of *in vitro* cell systems.

INTRODUCTION

Serious concern for the possible effects of genetic toxicants in the environment developed approximately a decade ago with the discovery of chemical mutagens capable of inducing high frequencies of mutation at high levels of survival (17). Concern also stemmed from the realization that man was greatly expanding the number of compounds theoretically capable of increasing mutation frequencies beyond present 'spontaneous' levels. As recently stated at an open meeting sponsored by the United States Department of Health, Education and Welfare on the value of selected test systems to detect and assess the mutagenic activity of chemicals (21), a human disease burden exists which is of genetic origin. Increases in mutation frequency can be expected to enlarge this burden, and many classes of chemicals already in the environment are known to include genetic toxicants. Although uncertainty remains regarding the precise impact such compounds might have upon human health, there is justification for apprehension (15).

Genetic toxicology, a new branch of toxicology concerned with the identification and evaluation of DNA-damaging agents (carcinogens, mutagens and some teratogens), may be broadly divided into (a) screening tests for identification of potential toxicants, (b) procedures for estimating risk, and (c) techniques for population monitoring. Screening involves primarily the use of rapid, inexpensive assays which detect agents capable of damaging or altering DNA. Because DNA is chemically and structurally similar in most organisms, and is considered the probable target of genetic toxicants, any organism or appropriate part thereof may be theoretically employed as a screening tool. Accordingly, viruses (18), a variety of microbial systems (3,36), cultured animal cells (14, 39, 26), *Drosophila* (2, 48), and various subcellular assays designed to measure effects directly on DNA (47, 49) are widely used for screening purposes. Several short-term tests utilizing intact mammals are also available for screening (31).

This paper is concerned with the application of an *in vitro* mammalian cell assay utilizing nutritional markers as an indicator system for genetic toxicants detected as mutagens. Major objectives are to (a) outline techniques for measuring the acute toxicity of chemicals to cultured cells, (b) qualitatively describe the CHO Cell/BrdU-VL system as an assay for mutation, (c) present data relative to the mutagenic potential of a series of compounds known to accumulate in the tissues of edible marine organisms (41, 45, 52) or which have been associated with the occurance of neoplasias in such organisms (8, 51), and (d) illustrate some additional end points, as well as some potential problems, pertinent to the application of *in vitro* cell assays. A detailed description of equipment, reagents, special techniques and experimental procedures relevant to the CHO Cell/BrdU-VL system will not be given here. A general protocol for this assay has been published by Kao and Puck (28). Our modifications to their procedure will be described elsewhere (33).

METHODS

Acute toxicity

Before compounds can be evaluated for mutagenic activity, their acute, physiological toxicity must be determined. This is accomplished by measuring the ability of single cells to produce macroscopic colonies arising in experimental dishes following exposure to specific concentrations of the test agent for specified periods of time. Relative plating efficiency (RPE), defined as the ratio of macroscopic colonies arising in experimental dishes, to those appearing in controls, may be plotted against dose to yield survival curves of the type shown in Figure 7-1. Concentrations of compound to be tested for genetic activity are selected from such curves. The exponential portion of each curve is described by equation [1] where (S/S_0) is the surviving cell fraction or percent RPE, (n) is the hit or target number (30) and (D/D_0) is relative dose (44). The target number is defined operationally by the intersection of the exponential portion of the curve with the ordinate axis when the former is extrapolated back. Relative dose is defined as the ratio of the experimental molar concentration of toxicant (D) to that increase in molar concentration (D_0) required to reduce the cell population by the fraction (l/e). The value of D_0 is, for each compound, obtained from a plot of molar concentration versus surviving cell fraction. Because chemicals differ in their molar toxicity by orders of magnitude; relative dose provides a convenient way to depict survival data for many compounds simultaneously. It is noted that the random hit model expressed by equation [1] was derived for radiation effects (30), and requires interpretive modifications when describing cell inactivation by chemicals (32). The use of plating efficiency to assess acute chemical toxicity has been described elsewhere (34).

$$\frac{S}{S_0} = \left[1 - \left(1 - e^{-D/D_0}\right)^n\right] \qquad [1]$$

The CHO Cell/BrdU-VL System

Figure 7-2 represents a simplified and generalized protocol for inducing, isolating and characterizing mutant cells. Initially, cells are inoculated into dishes or flasks and allowed to attach to the plastic substratum. Following attachment, cells are exposed to the test agent at one or more concentrations. The cells are then washed free of the test compound and fresh medium added. During the expression period, cells are grown under nonselective conditions, permitting induced genetic damage to become fixed into DNA, and ultimately to become expressed at the cellular level. The length of the expression period is a function of the system employed, the genetic markers involved, and the conditions of the experiment. Selection represents the application of a set of conditions permitting mutant cells to survive while eliminating wild-type cells. Selective conditions employed are specific for the type of mutant sought. Once potential mutants have been isolated, they may be subjected to genetic analysis for confirmatory purposes and for further characterization.

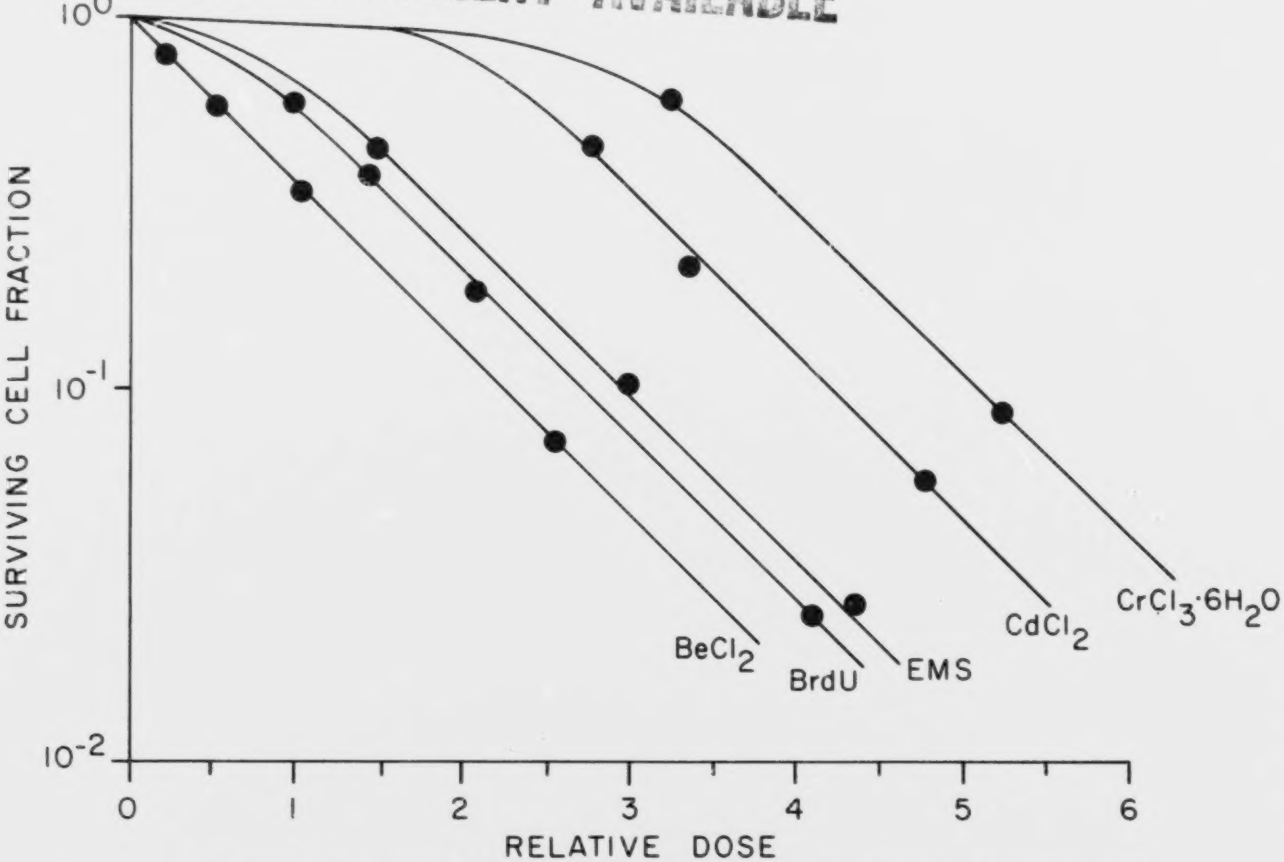

Figure 7-1. Typical 16-hour survival curves for five test compounds.

Note: The curves are constructed from relative plating efficiency data by plotting the surviving cell fraction (S/S_o) against relative dose (D/D_o). Concentrations or doses of toxicant employed in mutagenesis experiments are selected from such curves.

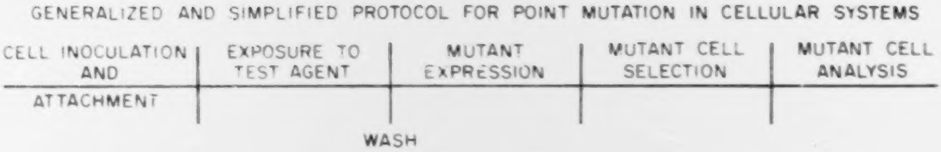
GENERALIZED AND SIMPLIFIED PROTOCOL FOR POINT MUTATION IN CELLULAR SYSTEMS
CELL INOCULATION AND ATTACHMENT
EXPOSURE TO TEST AGENT
MUTANT EXPRESSION
MUTANT CELL SELECTION
MUTANT CELL ANALYSIS
WASH

Figure 7-2. A generalized and simplified protocol for the induction, isolation and characterization of mutations in cellular systems.

Figure 7-3 represents a protocol containing the same basic features as that in Figure 7-2, but is specific for the CHO Cell BrdU-VL system. In this technique, CHO-K1 cells are cultured in two types of media. One medium (F12D) contains the minimal nutritional requirements for the growth of single cells into macroscopic colonies with high efficiency. A second, enriched medium (F12) is constructed from the minimal by addition of nine nutrilites (alanine, glycine, aspartic acid, glutamic acid, lipoic acid, vitamin B_{12}, inositol,

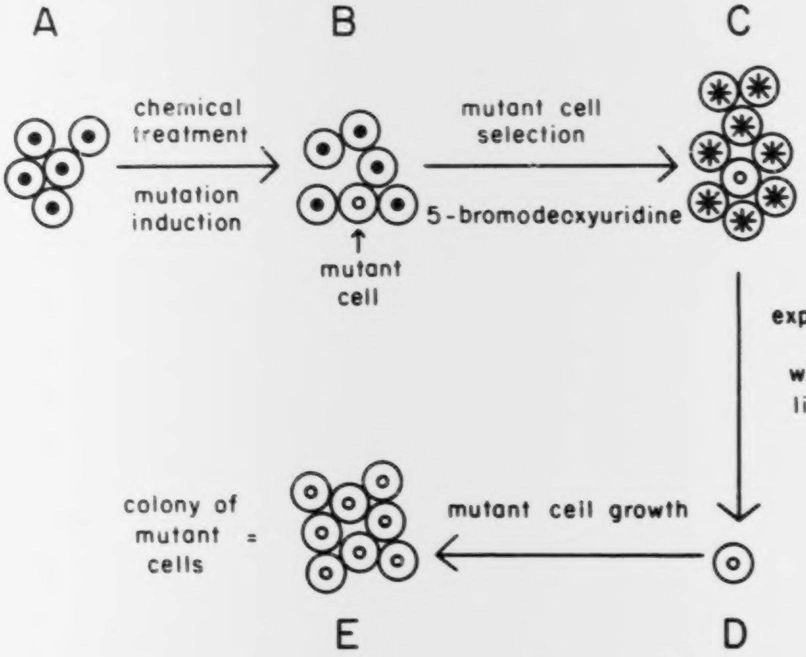
A
B
C
chemical treatment
mutation induction
mutant cell
mutant cell selection
5-bromodeoxyuridine
exposure to white light
colony of mutant cells =
mutant cell growth
E
D

Figure 7-3. Schematic representation of the Chinese hamster ovarian cell system (BrdU-visible light selection procedure) for the detection of small-scale mutations.

Note: (A) Population of wild-type cells. (B) Nutritional mutant (auxotroph) following induction and expression. (C) Wild-type cells with BrdU-containing DNA. (D) Surviving mutant cell following selective elimination of wild-types via the combined action of BrdU and white light. (E) Colony of mutant cells which grew in enriched medium. (After Kao and Puck (28)).

thymidine and hypoxanthine) not required exogenously by the cells for optimal growth. Mutants are detected by screening populations of cells exposed to test compounds for nutritionally deficient forms (auxotrophs) requiring one or more of the nine nutrilites omitted from F12D medium.

Operationally, 10^7 cells are exposed in four parallel cultures to single or multiple doses of the test agent, following inoculation and cell attachment (point A, Figure 7-3). Because even induced mutation is a rare event, there will generally be, following expression, a small number of mutant cells growning among millions of nonmutants in enriched medium. To identify the mutants, it is necessary to introduce a procedure which will eliminate the prototrophic (wild-type) cells, while allowing auxotrophs to survive. This is accomplished by taking advantage of the fact that mutants auxotrophic for one or more of the nine nutrilites omitted from F12D medium will be unable to grow in this medium, whereas wild-types will. Thus, at point B, Figure 7-3, F12 medium is replaced with F12D medium. This initiates the selective process by effectively terminating protein and nucleic acid synthesis. The thymidine analog, BrdU, is then added to the F12D medium from which it is incorporated into the DNA of wild-type cells (point C, Figure 7-3). Subsequent illumination of the cell population with white light is lethal to those cells having incorporated sufficient BrdU. Mutant cells do not incorporate BrdU, and survive the selective process (point D, Figure 7-3). Wild-type cells survive selection to an extent approaching 0.02 percent. In the presence of F12 medium, mutant cells, along with some wild-types, grow into macroscopic colonies (point E, Figure 7-3). These are picked and tested for mutant identification. It is important to note that the use of a known mutagenic agent (BrdU) in the selective process is of no consequence, as the only cells incorporating BrdU are wild-types destined for death. Mutants to be isolated are existent in the population at the end of the expression period prior to the application of selective conditions.

The procedure illustrated in Figure 7-3 and described above was applied in collecting the mutagenesis data presented below. Figure 7-4 shows the lighting apparatus used to illuminate cell populations following exposure to BrdU. Figure 7-5 shows cell survival in four randomly selected dishes several days after illumination. Cells not killed in selection have grown into macroscopic colonies. Because mutant and wild-type cells differ only in their requirements for exogenous nutrilites, they may be distinguished only by analysis of their growth properties in enriched and deficient media or by biochemical analysis.

Statistical Analysis of Data

When testing compounds for mutagenic activity, we usually want to know if the number of mutants per unit number of viable cells screened is sufficiently larger in experimental, versus control situations, to support a conclusion of

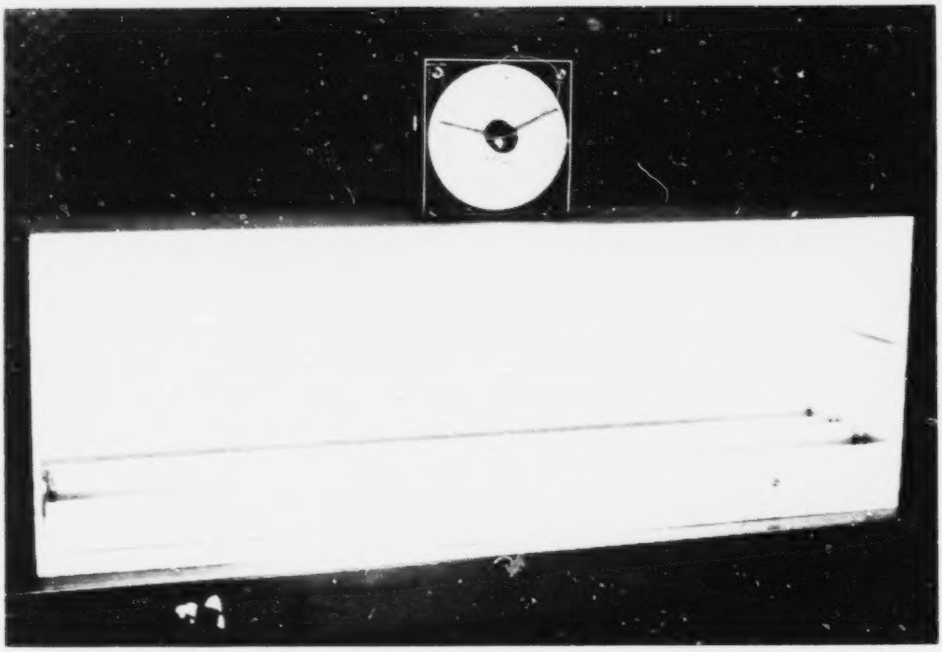

Figure 7-4. Illumination apparatus for the inactivation of cells with BrdU-containing DNA.

Note: Plastic culture dishes containing 2×10^4 cells each are subjected to white fluorescent light for 60 minutes.

Figure 7-5. Macroscopic colonies in four randomly selected dishes approximately seven days after illumination with white light.

Note: These clones, originating from cells surviving selection, are tested to determine if any are auxotrophic mutants.

induced mutation by the test compound. In the CHO Cell/BrdU-VL assay, each cell screened will either be auxotrophic for one or more of the nine nutrilites omitted for F12D (operational definition of mutant), or it will not be (operational definition of wild-type). The actual criteria employed to classify mammalian cell variants as true mutants are somewhat complex, and have been reviewed recently (16, 40, 46). For the purpose of this paper, the operational definitions given above shall be used.

In the standard procedure, a sample of 10^6 cells from each test or control population is distributed among fifty 60 mm dishes, and subjected to the selective process. Surviving clones are then sampled and tested for the presence of auxotrophs. The total number of auxotrophs expected per 10^6 viable cells is then estimated from the data by equation [2], where (y) is the estimated number of auxotrophs, (x) is the number of auxotrophs observed, (n) is the number of replica experiments, (A) is the total number of cells surviving selection, (B) is the total number of colonies picked and tested, (C) is the initial number of 60 mm dishes, (D) is the final number of dishes (some may be lost to contamination during the course of the experiment), and (E) is the absolute plating efficiency (defined as the ratio of macroscopic colonies produced to cells inoculated) as measured in low density control dishes. Because mutants are randomly distributed among wild-types in mixed populations, the probability that any given survivor will be a mutant should be constant over all survivors. Moreover, as only a small number of mutants is generally found in any given population, the distribution of mutants in such populations should be Poisson. Accordingly, mutagenesis data from sets of replica experiments were tested for goodness of fit to a Poisson model and found to be consistent with this type of distribution (33).

For two independent Poisson variables (X, Y), a new statistic (V) has been proposed by Best (9) for testing the difference between two Poisson expectations (e.g., the estimated mean number of mutants in experimental (X) versus control (Y) populations). This statistic, given by equation [3], is similar in performance to the more familiar square root of the Poisson Index of Dispersion (20), except in the tails of the distribution where (V) is superior. Although (V) is a function of the Poisson variables (X, Y), (V) itself shows an approximately normal distribution. This statistic may be particularly applicable to mutagenesis data where the difference in variance observed between experimental and control populations is large. This is the situation at the present time with the CHO Cell/BrdU-VL system where mutants are rarely observed in control populations. All mutagenesis data considered below were scaled via equation [2] and compared to an historical control (Y) in accordance with equation [3] and appropriate confidence limits. The model given by equation [3] is at present a proposed one and may not be the final model of choice.

$$(y) = (x/n)\,[(A)/(B)]\,[(C)/(D)(E)] \qquad [2]$$

$$(V) = (2X + 3/4)^{1/2} - (2Y + 3/4)^{1/2} \qquad [3]$$

RESULTS

Control Investigations

With the exception of the spontaneous proline auxotroph isolated as the K1 subclone of the CHO cell (44), other spontaneous auxotrophs with requirements for one or more of the nutrilites omitted from F12D medium had not been previously reported for the CHO Cell/BrdU-VL system. Such auxotrophs could easily be suppressed in stock cell populations by maintaining cells in minimal rather than enriched medium. This was not done in order to determine if spontaneously arising auxotrophs could indeed be identified in control or stock populations. Table 7-1 summarizes data from 16 different control experiments carried out over a period of several months. Two glycine mutants were identified among 989 clones picked and tested. The observed frequency of spontaneous auxotrophy is thus two mutants per 1.2×10^7 viable cells. These data, in combination with data for the other parameters of equation [2], were utilized to obtain an estimate of 0.331 mutants per 10^6 viable control cells. This value was substituted for (Y) in equation [3].

Table 7-1. Summary Mutagenesis Data From Several Control Experiments

(n)	Viable cells	[(A)/(B)]	[(C)/(D)(E)]	Auxotrophys isolated Gly	Hyp	(x)	(y)
16	1.2×10^7	1888/989	(800)/(770) (.750)	2	0	2	0.331

The scaling of (x) by equation [2] does not consider the fact that mutant cells may be lost to the effects of starvation during selection. In fact, reconstruction experiments employing known numbers of mutants have demonstrated that this type of loss does occur for the three types of auxotrophs observed (27, 33). Consequently, equation [2] underestimates actual mutant frequencies.

Induced Mutation with Standard Mutagens

The standard mutagens, EMS and BrdU, were evaluated for mutagenic activity at several doses. Data pertinent to the mutagenicity of these compounds are presented in terms of the parameters of equation [2] in Table 7-2. The estimated number of mutants per 10^6 viable cells (7) is plotted in Figure 7-6 as a function of relative dose. By expressing the mutagenesis data in terms of mutant cell frequencies per 10^6 viable cells, meaningful comparisons between different compounds or different doses of the same compound could be made. Applying the data to equation [3], EMS was mutagenic at all doses tested ($\alpha < 0.05$). BrdU was mutagenic at five of seven doses tested, and produced a complex dose-response pattern similar to those observed with hycanthone methanesulfonate, and other compounds in different assays (12, 13).

Induced Mutation with Other Compounds

Forward mutation experiments employing doses of toxicant generally yielding 20 percent survival or greater were carried out in replica with several inorganic compounds, and an aqueous extract of JP-5 jet fuel. The data are presented in Table 7-3 in terms of the parameters of equation [2]. These compounds or mixtures were selected for evaluation as mutagens because they were either known to have, or were suspected of having, carcinogenic properties. Their mutagenic response in the CHO Cell/BrdU-VL system can be divided into three classes: (1) Experiments with the oxides of arsenic and selenium, lead acetate, and the chloride salts of cobalt and nickel, failed to produce any auxotrophs; (2) tests with beryllium and chromium usually produced auxotrophs, but never in sufficient numbers to support a conclusion of induced mutation; (3) Experiments with cadmium chloride, manganese chloride, and an aqueous extract of JP-5 jet fuel, also produce auxotrophs, sometimes in sufficient numbers to suggest induced mutation by these compounds. As indicated in Table 7-3, it was possible to obtain relatively large numbers of auxotrophs with $CdCl_2$. Usually, however, observed mutant frequencies were low. Cadmium chloride was found to be significantly mutagenic in about 20 percent of experiments, as was the extract of JP-5 jet fuel. Manganous chloride was observed to be mutagenic in approximately 50 percent of experiments.

Isolation of Nonauxotrophic Variants

Two classes of variants, other than auxotrophs, were isolated from cell populations treated with known genetic toxicants. One class consists of cells exhibiting a rounded morphology, and represents cells unable to stretch out on

Table 7-2. Data From Forward Mutation Experiments With EMS and BrdU

Experiment	Molarity (D/D$_o$)	(n)	Viable Cells	[(A)/(B)]	[(C)/(D)(E)]	Auxotrophs isolated			(y)
						Gly	Hyp	(x)	
1 (EMS)	1.50×10^{-3} 2.17	4	2.5×10^{6}	339/138	(200)/(183)(0.625)	3	8	11	11.8**
2 (EMS)	1.75×10^{-3} 2.54	4	2.4×10^{6}	605/173	(200)/(180)(0.603)	8	3	11	17.6**
3 (EMS)	3.00×10^{-3} 4.35	4	2.8×10^{6}	436/163	(200)/(200)(0.703)	44	2	46	43.8**
1 (BrdU)	7.00×10^{-5} 1.42	4	1.6×10^{6}	949/361	(200)/(180)(0.400)	2	1	3	5.5*
2 (BrdU)	1.00×10^{-4} 2.45	1	1.2×10^{5}	169/081	(050)/(050)(0.120)	4	1	5	86.9**
3 (BrdU)	1.30×10^{-4} 3.62	1	1.6×10^{5}	161/046	(050)/(034)(0.160)	0	0	0	0.0
4 (BrdU)	2.40×10^{-4} 4.90	1	2.1×10^{5}	248/127	(050)/(050)(0.210)	0	0	0	0.0
5 (BrdU)	3.50×10^{-4} 7.14	1	3.2×10^{5}	326/145	(050)/(048)(0.320)	2	1	3	22.1**
6 (BrdU)	4.60×10^{-4} 9.38	1	2.8×10^{5}	214/128	(050(/(050)(0.280)	1	4	5	30.1**
7 (BrdU)	5.70×10^{-4} 11.63	1	7.2×10^{5}	160/109	(050)/(049)(0.720)	6	1	7	14.5**

* Denotes significance at the 95% confidence level; ** denotes significance at the 99% confidence level.

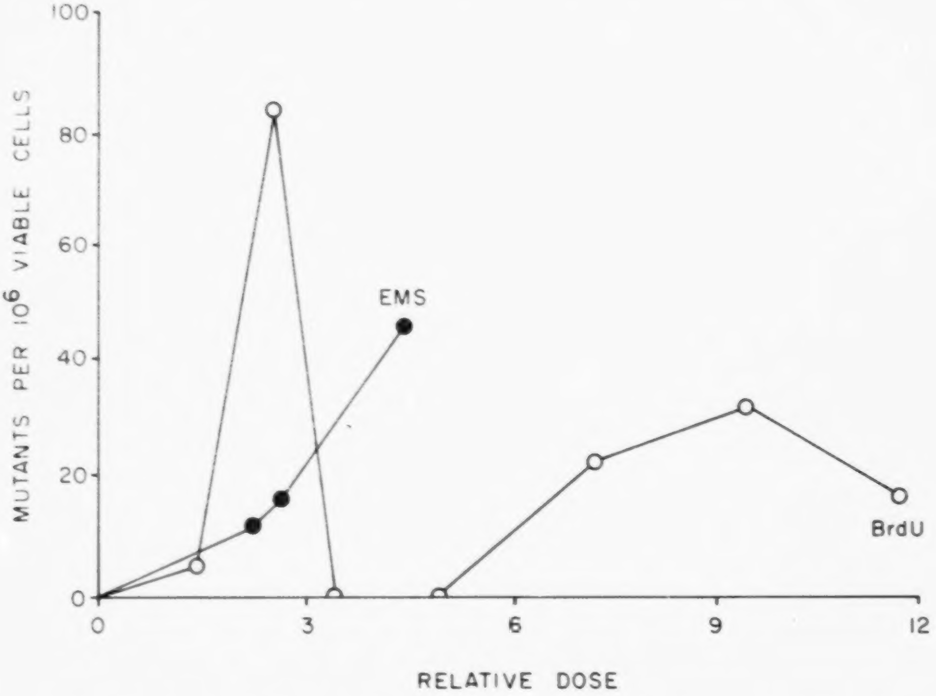

Figure 7-6. Mutation frequency induced by EMS and BrdU as a function of dose.

the plastic substratum and assume the standard epithelial morphology (Figure 7-7). Although this type of cell produces colonies of sufficient size to permit cloning, the cells are continuously in a rounded state, as if entering mitosis. When grown in medium containing 10 percent fetal calf serum, some of the clones assume a more normal morphology, and may represent mutants with increased serum requirements (27). To date, this type of variant has been observed only in populations treated with EMS, BrdU, and compounds of chromium, cadmium and lead.

A second type of variant, appearing to possess the property of contact inhibition (1, 19), was isolated from cell populations treated with the known carcinogens, EMS, CrO_3 and $PbAc_2 \cdot 3H_2O$. These were detected as wild-type cells surviving selection, and which possessed a pronounced fibroblastic morphology (Figure 7-7). When cells were grown into confluent monolayers, unlike the transformed CHO-K1 cell, they ceased to divide and assumed a state of contact inhibition, or a state resembling that of contact inhibition. Dense monolayers remained for as long as two weeks without medium changes, and without significant deterioration. Confluent sheets of cells could be easily trypsinized and dispersed into uniform, single-cell populations. Upon replating in fresh growth medium, cells grew with a generation time of approximately 14 hours, ceasing to divide when the monolayer again became confluent.

Table 7-3. Data From Forward Mutation Experiments with Selected Additional Compounds

Chemical	Molarity	(n)	Viable	[(A)/(B)]	[(C)/(D)(E)]	Auxotrophs isolated			(y)
	(D/D_o)		Cells			Gly	Hyp	(x)	
As_2O_3	3.0×10^{-5} 3.85	4	3.1×10^6	541/250	(200)/(192)(0.770)	0	0	0	0.0
$BeC1_2$	5.0×10^{-4} 2.50	4	3.2×10^6	680/230	(200)/(200)(0.865)	2	0	2	1.7
$CdC1_2$	5.0×10^{-7} 4.73	4	2.5×10^6	1347/162	(200)/(173)(0.615)	36	0	36	140.7**
CrO_3	7.0×10^{-6} 3.68	4	2.2×10^6	253/188	(200)/(166)(0.549)	2	0	2	1.5
$CrC1_3 \cdot 6H_2O$	3.0×10^{-3} 3.15	4	3.1×10^6	623/304	(200)/(163)(0.780)	1	0	1	0.8
$CoC1_2 \cdot 6H_2O$	1.5×10^{-4} 1.98	4	2.1×10^6	168/090	(200)/(189)(0.525)	0	0	0	0.0
$NiC1_2 \cdot 6H_2O$	2.0×10^{-4} 2.56	4	3.2×10^6	458/238	(200)/(200)(0.800)	0	0	0	0.0
$MnC1_2 \cdot 4H_2O$	3.5×10^{-4} 3.37	4	2.5×10^6	769/357	(201)/(191)(0.618	4	0	4	3.7*
$PbAc_2 \cdot 3H_2O$	5.0×10^{-4} – – –	4	3.2×10^6	592/359	(201)/(201)(0.800)	0	0	0	0.0
SeO_2	1.5×10^{-5} 0.26	4	2.3×10^6	276/156	(200)/(180)(0.575)	0	0	0	0.0
JP-5	Aq. extract 1:15 dilut.	4	2.9×10^6	892/391	(200)/(187)(0.738)	7	0	7	5.8*

* Denotes significance at the 95% confidence level; ** denotes significance at the 99% confidence level.

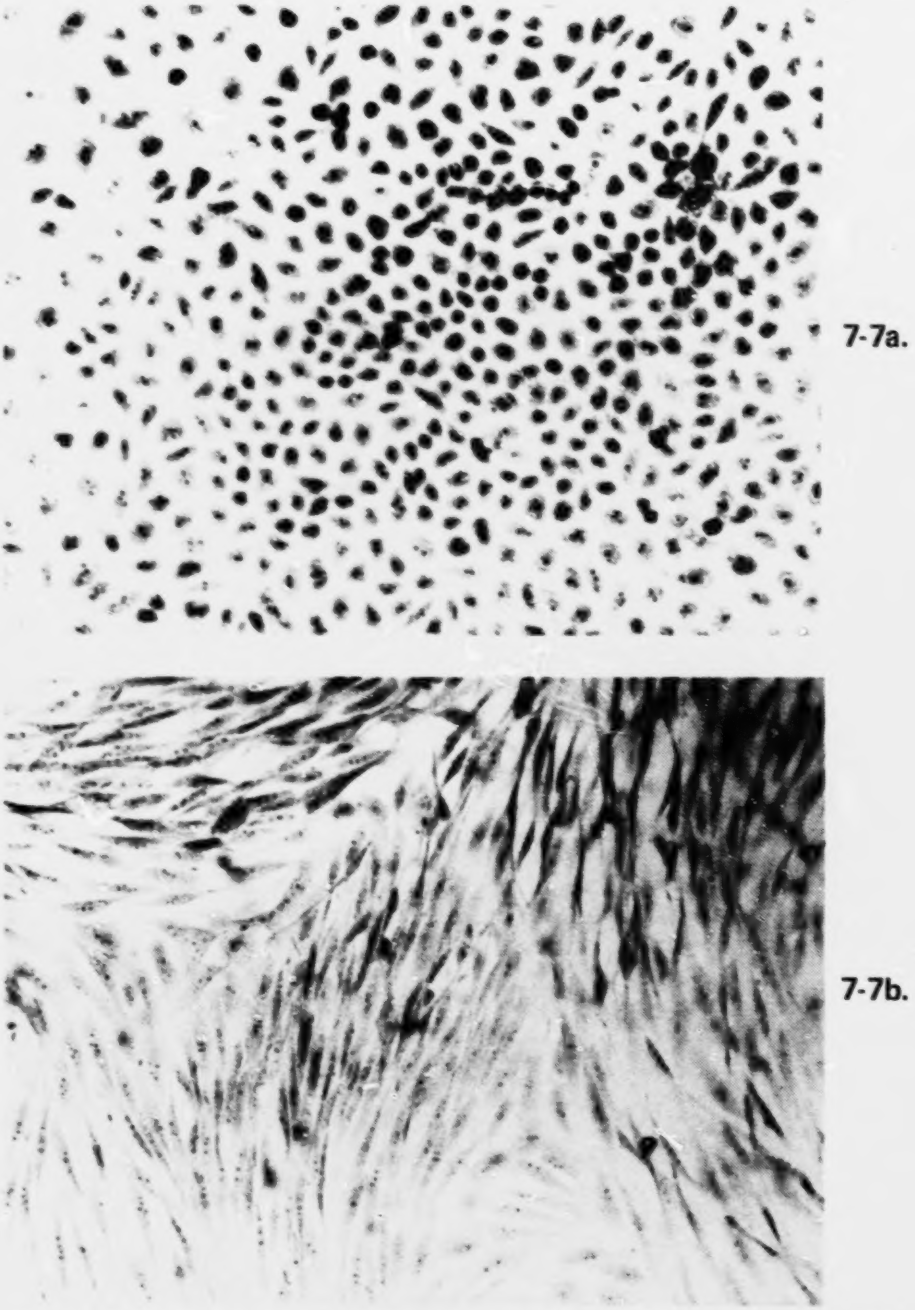

Figure 7-7. Epithelial and Fibroblastic Morphologies Exhibited by the CHO-K1 Cell.

Note: (A) shows the standard epithelial morphology usually adopted by the cell. (B) illustrates the highly fibroblastic form assumed by a potentially contact-inhibited cell isolated from a population treated with the known carcinogenic agent chromium trioxide.

Preliminary experiments suggest that these cells show contact inhibition of replication in colonies containing at least 100 cells, as well as in dense monolayers (35). All such clones tested to date appear stable in their ability to express the apparent contact-inhibited state.

Mutation Frequency and Expression Time

Mutation is a complex biological process involving much more than interaction of mutagen with DNA, or more generally, with the DNA-replicating system. The mutagen-DNA interaction usually results not in mutation, but in the creation of premutational lesions which become expressed as mutations only after a series of additional events has occured (6). For example, auxotrophic mutants usually become expressed as soon as the cells become depleted of normal gene product. The length of time required for this and other preliminary events to occur may vary with the nature of the gene product, the specific mutagen utilized, and the doses employed (5, 7). To determine the influence of expression time on observed mutation frequency for the mutant phenotypes reported above, experiments employing variable expression time were carried out with EMS and BrdU. Doses used were those giving maximal observed mutation frequency when the expression time was five days (3×10^{-3} molar for EMS, 1×10^{-4} for BrdU). Expression time was varied between two and eleven days. The data are given in Table 7-4, again in terms of the parameters of equation [2]. Observed mutation frequency is plotted in Figure 7-8 as a function of expression time.

Optimal expression time is defined as the interval between mutagen quenching, and the application of selective conditions yielding maximal mutation frequency when dose is held constant. This is observed to be five days for BrdU and eight days for EMS. Moreover, for a two-day expression period, statistically significant numbers of mutants could always be identified in populations exposed to EMS, whereas none are found in populations treated with BrdU. Once optimal expression time is exceeded, mutant cell frequency decreased rapidly, suggesting that these types of mutants are at a replicative disadvantage relative to wild-type cells under nonselective conditions. The fact that optimal expression time was different for the two mutagens when tested at doses showing similar toxicity, suggests that expression time may be mutagen dependent. This lends support to the contention that assays utilizing variable expression time shall be required when screening compounds for mutagenic activity (4). This may be particularly important for weak mutagens.

DISCUSSION

The genetic toxicology of inorganic compounds has largely been ignored, despite the fact that many are ubiquitous, highly toxic, and implicated as

Table 7-4. Auxotroph Frequency as a Function of Expression Time for Single Doses of EMS and BrdU

Chemical	Expression (days)	(n)	Viable cells	[(A)/(B)]	[(C)/(D)(E)]	Auxotrophs isolated				(y)
						Gly^-	Hyp^-	GHT^-	(x)	
EMS	2	1	1.2×10^5	109/050	(051)/(051)(.120)	3	0	1	4	072.7**
	5	1	3.4×10^5	368/143	(051)/(049)(.338)	12	6	1	19	149.9**
	8	1	5.9×10^5	408/147	(051)/(051)(.593)	41	5	0	46	213.5**
	11	1	6.1×10^5	531/105	(051)/(051)(.610)	11	1	0	12	99.5**
BrdU	2	1	1.3×10^5	086/040	(051)/(032)(.125)	0	0	0	0	0.0
	5	1	5.0×10^4	180/113	(051)/(045)(.005)	3	0	0	3	108.1**
	8	1	6.3×10^5	569/107	(051)/(043)(.633)	3	1	0	4	39.1**
	11	1	5.8×10^5	414/075	(051)/(049)(.578)	1	1	0	2	6.8**

Doses: EMS; 3.0×10^{-3} molar ($D/D_o = 4.35$). BrdU; 1.0×10^{-4} molar ($D/D_o = 2.04$).

GHT^- designates an auxotroph with simultaneous requirements for glycine, hypoxanthine and thymidine.

** Denotes significance at the 99% confidence level.

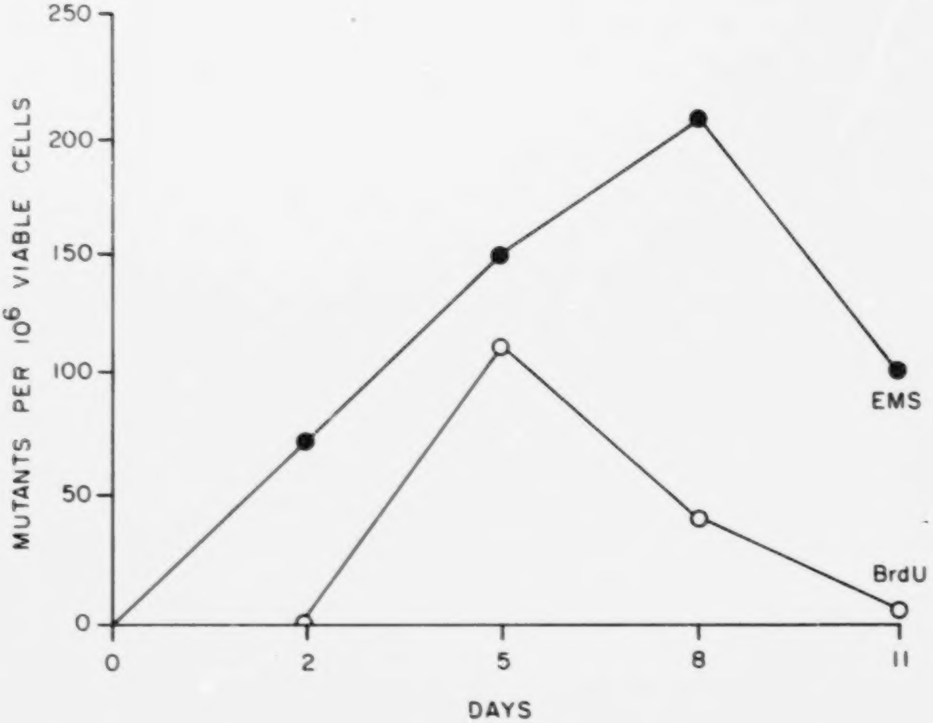

Figure 7-8. Mutation frequency induced by EMS and BrdU as a function of expression time.

human carcinogens. Neglect of the inorganics in this regard seems due, in part, to the fact that such compounds comprise a relatively small percentage of known or potential genetic toxicants, are difficult to handle in many experimental situations, appear to mediate genetic effects by obscure mechanisms, and have not been active in many major assays. Some metals have shown activity in DNA repair tests employing microbial systems (37), and in reverse mutation assays with *E. coli* (50). Recently, selenate and some compounds of chromium have been shown to be active in selected strains of *Salmonella typhimurium* (38). Perhaps the best assay developed to date for predicting the potential genetic toxicity of metals is the *in vitro* DNA-synthesizing system described by Sirover and Loeb, and which detects copying errors in replicating DNA (47). The fact that the carcinogenic metals were not generally active in the CHO Cell/BrdU-VL system may reflect a sensitivity problem related to the loss of mutants during selection to the effects of starvation. Because mutation is periodically observed with certain of these compounds, factors other than assay sensitivity may be involved. These factors could be uniquely important to the expression of mutagenic activity by metals and may not be presently under control or consideration. Extensive testing with cadmium chloride and beryllium chloride indicates that the problem does not lie with expression time.

The isolation of potentially contact-inhibited cells from populations treated with known carcinogenic agents is interesting for several reasons. Among the more intriging ideas is the possibility that such cells represent back transformation to the noncancerous state. The CHO cell possesses many of the properties of transformed cells, including the loss of contact inhibition (42, 44). In accordance with the somatic cell mutation hypothesis for cancer (10, 22), agents inducing cell transformation through mutation should, in at least some cases, be capable of inducing back transformation in individual cells via true reverse mutation or via forward mutation at suppressor loci. Indeed, spontaneous revertants of cells transformed to the malignant state *in vitro* by viruses and chemicals have already been described (23, 24). Reversible conversion of transformed cells to the contact-inhibited state has been observed during exposure of such cells to dybutryl cyclic AMP (25) or concanavalin A (11). It remains to be shown if the same agents capable of inducing transformation form normal to malignant state can also reverse it, such that the revertants are stable in the absence of inducing agent. Although virtually all cell-transforming agents tested to date are also mutagens, it is not yet possible to say if the phenomenon of cell transformation involves mutation (29). Investigation of the properties of these cells is continuing.

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DEVELOPMENT OF A BIOASSAY FOR OILS USING BROWN ALGAE

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ABSTRACT

Bioassay procedures were developed to observe the effects of No. 2 fuel oil, two jet fuels, and a crude oil on the growth and early development of *Fucus* zygotes and *Laminaria* gametophytes. These algae are as sensitive, or more so, than fish and invertebrates previously tested in oil bioassays. *Fucus* sperm and *Laminaria* spores are extremely sensitive to oil, with dramatic effects at the levels of 2 ppb. These results indicated that these species are potentially good bioassay organisms, and also that chronic, low-level pollution could significantly alter the community structure in marine ecosystems.

INTRODUCTION

Algae are the primary producers in the marine ecosystem. Yet, despite their importance, little is known on how they are affected by specific pollutants in their environment. A bioassay is one method of studying pollution effects on organisms. This paper reports on bioassay procedures developed with two brown algal genera, *Fucus* and *Laminaria*.

In order to be a useful bioassay organism, an alga should be readily available in nature, easily maintained in the laboratory, hardy enough to grow in culture, yet sensitive enough to respond to low levels of pollution encountered in nature, and preferably, of ecological or economic importance. Both *Fucus* and *Laminaria* have most, if not all, of these qualities; yet, because of differences in their habitats, they might react differently to an oil spill. *Fucus*, being an intertidal species, would be subjected to repeated immersion in the water mass and be coated with oil when the tide is out. *Laminaria* is a subtidal species and would normally be subjected only to concentrations of oil present in the water column.

Although *Fucus* and *Laminaria* are both brown algae, their life cycles are quite different. The life cycle of *Fucus* (Figure 8-1) is very much like that of an animal. Mature, diploid thalli produce haploid eggs and sperm which fuse to form zygotes that ultimately develop into new diploid thalli. The life cycle of *Laminaria* (Figure 8-2), in which an alternation of generations occurs, is much more complicated than that of *Fucus*. The macroscopic, blade-like, diploid sporophyte alternates with microscopic, filamentous, haploid gametophytes. The variability and complexity of algal life cycles provide several opportunities to study the effect of a specific pollutant on growth and development. In the present study, the effects of different petroleum products on the growth of *Fucus* zygotes and *Laminaria* gametophytes were observed.

MATERIALS AND METHODS

Both *Fucus* and *Laminaria* plants were collected from various locations in and around Narragansett Bay (i.e., Camp Varnum, a National Guard installation, the dock of the Environmental Research Laboratory, Narragansett, R.I., and Monohan's Cove, Narragansett, R.I.). In developing techniques, several species were used, including *Fucus vesiculosus*, *F. edentatus*, *F. distichus*, *Laminaria saccharine*, and *L. digitalis*.

The *Fucus* species represent both monoecious and dioecious types. In deciding which species of *Fucus* and *Laminaria* to use, little difference was noted in preliminary response among various species. Data presented herein represent the responses of *Fucus edentatus* and *Laminaria saccharina*, but are representative of other species in both genera.

For *Fucus*, methods of procurement of eggs and sperm were evaluated (4, 5), and a technique was devised that is applicable to all species tested. The method is essentially a combination of other methods reported in the literature, and consists of the following: receptacles (fertile plant tips) that appeared most erumpent and mature, even to the point of being partially eroded, were collected from mature plants. These receptacles were observed to produce the highest numbers of viable eggs and sperm. Receptacles were rinsed in sterile charcoal filtered* seawater at 30 ppt. salinity, and were placed in a moist chamber overnight. The moist chamber consisted of large 150 × 25 mm plastic petri dishes (Falcon Plastics) containing filter paper of the same diameter moistened with sterile seawater.

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* Cartridge filtration through Commercial Filter Corporation honeycomb wound filters, 15 μ porosity, and .22 μ porosity pleated Gelman filters.

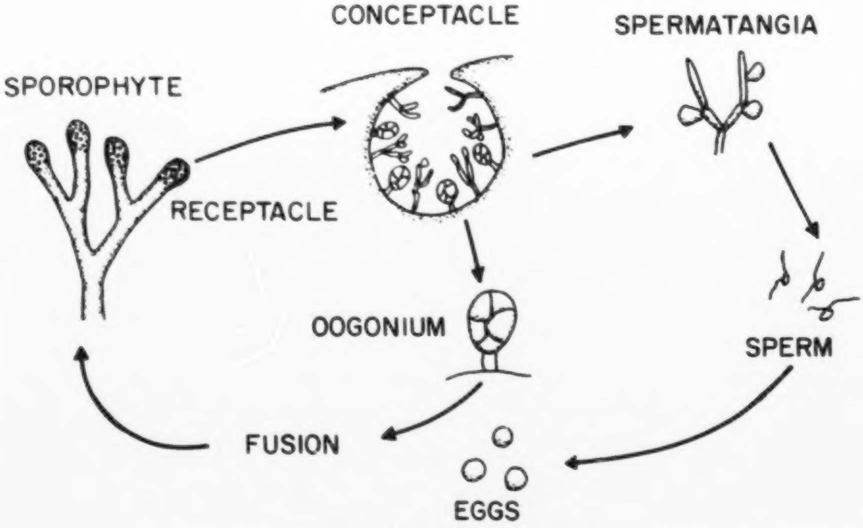
CONCEPTACLE
SPERMATANGIA
SPOROPHYTE
RECEPTACLE
OOGONIUM
SPERM
FUSION
EGGS

Figure 8-1. The Life Cycle of Fucus.

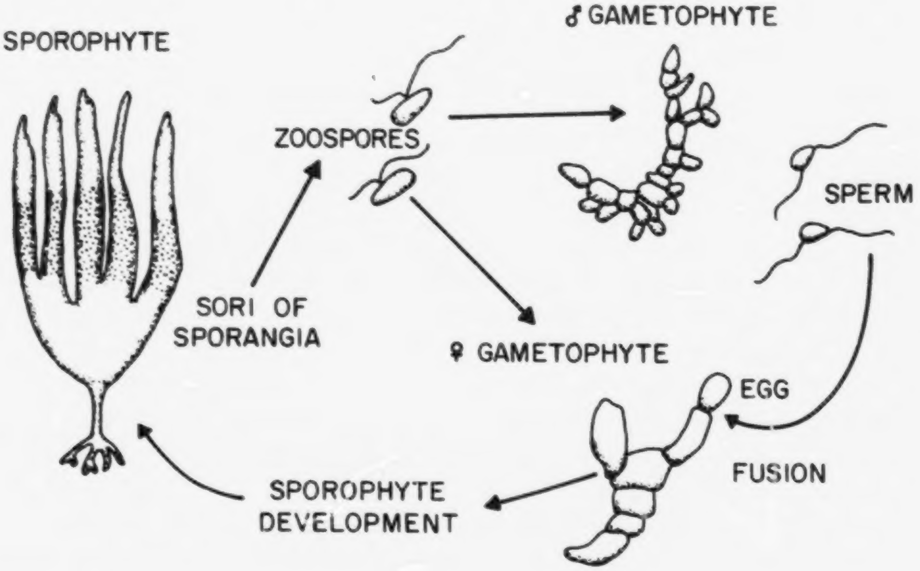
♂ GAMETOPHYTE
SPOROPHYTE
ZOOSPORES
SPERM
SORI OF SPORANGIA
♀ GAMETOPHYTE
EGG
FUSION
SPOROPHYTE DEVELOPMENT

Figure 8-2. The Life Cycle of Laminaria.

These plates were placed in a 12°C culture chamber overnight. Receptacles were then placed in sterile charcoal filtered seawater the next morning. Eggs and sperm were immediately released, and fertilization was observed within 15 to 20 minutes. Zygotes were immediately pipetted into culture dishes (60 x 15 mm plastic petri dishes, with 2 mm square grids – Falcon Plastics) while keeping the eggs suspended in seawater by stirring. Twenty four hours after dispensing into culture dishes, the toxicant was introduced by allowing the zygotes to settle on the bottom of the dish, the seawater removed, and replaced with seawater containing the toxicant at the test levels. In a few cases, the tips were pre-treated with the toxicant, and in these cases, the above sequence was suitably modified.

The growth medium for the *Fucus* assays was, in all cases, sterile charcoal filtered seawater. The parameter measured for these experiments was the increase in length after 12 days of growth.

Methods of procuring *Laminaria* spores were similar to those of *Fucus* gametes. Sporogenous plants were collected and then washed in deionized water to remove surface contaminants. Small pieces (2-3 cm square) of sporogenous tissue were placed into moist chambers overnight. These pieces were placed into sterile seawater the following morning, and spores were released in abundance. Spores were dispensed into culture dishes at concentrations sparse enough to allow counting and to prevent overcrowding, but dense enough (ca. 100-260 eggs) for good statistical data. In all cases, the culture medium was Provasoli's Enriched Seawater (6). The parameter measured was the increase in diameter of the gametophyte after 21 days of growth.

All assays were conducted at 400 ft-c of continuous cool white fluorescent light. Except for the first series of experiments to determine the optimal temperature salinity combinations for the assays, the temperature and salinity were 18°C and 30 o/oo for *Fucus* and either 12 or 18°C and 30 o/oo for *Laminaria*. For the various tests, the petroleum product (either No. 2 fuel oil, JP-4, JP-5, and Willamar crude) at concentrations ranging from 0-2000 ppm was equilibrated with seawater, proper dilutions made, and added to the cultures. The oil-seawater mixtures were analyzed by infrared spectrophotometry (Perkin-Elmer Model 621) to determine the amount of dissolved product causing toxicity.

Observations and measurements of *Fucus* zygotes and *Laminaria* gametophytes were made with a Unitron inverted microscope (Model BMIC). Approximately 10-20 individuals were measured per dish. Four replicates were performed for each treatment.

RESULTS

Fucus zygotes were much more tolerant of salinity-temperature extremes than were *Laminaria* gametophytes (Table 8-1). This is probably a reflection of their habitats; *Fucus*, being intertidal and growing in a more variable environment than the subtidal *Laminaria*, is adapted to a wider range of environmental conditions. Optimal growth of *Fucus* was at 18°C and 30-42 ppt while that of *Laminaria* was at 12–18°C and 24–36 ppt. There appeared to be some seasonal variability in *Laminaria*'s optimal temperature for growth. Fertile *Laminaria* collected during the colder winter months produced spores that germinated and grew slightly better at 12°C than at 18°C; the converse was true for *Laminaria* collected during the warmer spring months. In the following experiments to determine the toxicity of different oils, the standard conditions were 18°C and 30 ppt for *Fucus* and 12°C and 30 ppt for *Laminaria*.

Of the four types of oil tested, No. 2 fuel oil was the most toxic to *Fucus* zygotes, and the jet fuels, JP-4 and JP-5, were least toxic (Table 8-2). There appeared to be a slight stimulation at lower levels (200 ppb or less), except for Willamar crude. This may be due to a surfactant effect on the part of the oil. Above those levels, these oils became increasingly deleterious to growth.

The toxicity of the four oils to *Laminaria* gametophytes was similar to that of *Fucus* zygotes, although at lower levels, *Laminaria* response was not comparable to *Fucus* (Table 8-3). Number two fuel oil was still the most toxic, although not as relatively toxic as it was to *Fucus*. The jet fuels, JP-4 and JP-5, were somewhat more toxic to *Laminaria* than they were to *Fucus*. The lower growth rate of *Laminaria* gametophytes compared to *Fucus* zygotes is probably a reflection of their different growth habits. *Laminaria* gametophytes are much smaller, and grow in a more radial fashion than do *Fucus* zygotes.

Preliminary experiments on application of oil during gamete release in *Fucus*, and spore release in *Laminaria*, indicate that these brown algae may be extremely sensitive to oils (Table 8-4).

Concentrations greater than 20 ppb of No. 2 fuel oil were completely toxic to *Laminaria* spores. Even at 2 ppb, significant inhibition of the resulting gametophytes occurred. *Fucus* was even more sensitive. At 2 ppb, fertilization of eggs was blocked, apparently due to a toxicity effect on the sperm.

DISCUSSION

It is somewhat difficult to compare the results of these bioassays with brown algae, with those developed with other organisms by different

Table 8-1. Effects of Various Temperature and Salinity Combinations on the Growth of Fucus edentatus Zygotes and Laminaria saccharina Gametophytes.

Salinity, ‰	Length* of *Fucus* after 12 Days µm Temperature, C					Diameter* of *Laminaria* after 21 Days µm Temperature, C				
	6	12	18	24	30	6	12	18	24	30
6	102 ± 0	190 ± 23	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
12	153 ± 5	273 ± 15	341 ± 13	140 ± 0	0 ± 0	0 ± 0	0 ± 0	93 ± 7	0 ± 0	0 ± 0
18	165 ± 3	419 ± 13	586 ± 4	331 ± 28	0 ± 0	0 ± 0	102 ± 7	133 ± 8	0 ± 0	0 ± 0
24	151 ± 1	383 ± 9	645 ± 36	400 ± 13	0 ± 0	0 ± 0	168 ± 9	196 ± 8	0 ± 0	0 ± 0
30	153 ± 20	380 ± 3	704 ± 13	406 ± 9	0 ± 0	0 ± 0	265 ± 2	262	0 ± 0	0 ± 0
36	147 ± 17	395 ± 14	701 ± 18	400 ± 15	0 ± 0	0 ± 0	238 ± 8	184	0 ± 0	0 ± 0
42	122 ± 20	285 ± 14	712 ± 20	365 ± 17	0 ± 0	0 ± 0	88 ± 2	78	0 ± 0	0 ± 0
48	102 ± 13	260 ± 12	601 ± 68	342 ± 19	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
54	0 ± 0	254 ± 8	458 ± 16	295 ± 14	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
60	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

*Mean ± SE

Table 8-2. Effects of No. 2 Fuel Oil JP-4, JP-5 and Willamar Crude Oil on Growth of Fucus edentatus Zygotes.
Numbers Shown are μm in Length† of Juvenile Plants After 12 Days.

Added	Total Extractable Hydrocarbons	No. 2 Oil	JP-4	JP-5	Willamar Crude
0.0	*	694 ± 19	811 ± 48	811 ± 48	776 ± 13
2ppb	*	703 ± 6	758 ± 28	807 ± 31	764 ± 20
20ppb	*	748 ± 32	884 ± 37	913 ± 43	748 ± 7
200ppb	*	697 ± 9	818 ± 39	923 ± 37	750 ± 4
2ppm	(0-30ppb)	671 ± 21	854 ± 23	881 ± 24	735 ± 19
20ppm	(1-3ppm)	0 ± 0	781 ± 17	753 ± 4	656 ± 14
200ppm	(18-28ppm)	0 ± 0	655 ± 28	445 ± 22	399 ± 9
2ppt	(45-50ppm)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

* Dissolved hydrocarbons undetectable by spectrophotometry due to extremely minute amounts. Values are range of measurement and include all the petroleum products.

†Mean ± SE

Table 8-3. Effects of No. 2 Fuel Oil, JP-4, JP-5 and Willamar Crude Oil On Growth of *Laminaria saccharina* Gametophytes. Numbers Shown are μm in Diameter† Gametophytes After 21 Days.

Added	Total Extractable Hydrocarbons	No. 2 Oil	JP-4	JP-5	Willamar Crude
0.0	*	186.4 ± 2.3	184.0 ± 3.5	188.0 ± 2.5	198.0 ± 7.0
2ppb	*	158.0 ± 2.5	161.0 ± 2.5	152.0 ± 4.8	188.5 ± 4.7
20ppb	*	150.0 ± 5.4	148.2 ± 1.3	151.2 ± 5.7	194.2 ± 5.2
200ppb	*	136.1 ± 3.1	140.5 ± 1.2	132.2 ± 3.4	175.5 ± 5.6
2ppm	(0.30ppb)	120.2 ± 0.9	117.3 ± 2.1	129.2 ± 5.3	157.0 ± 5.9
20ppm	(1-3ppm)	105.9 ± 2.8	110.0 ± 2.3	102.8 ± 3.1	144.8 ± 3.2
200ppm	(18-28ppm)	**0 ± 0	**0 ± 0	0 ± 0	96.8 ± 2.2
2ppt	(45-50ppm)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

* Dissolved hydrocarbons undetectable by spectrophotometry due to extremely minute amounts. Values are range of measurement and include all the petroleum products.

** Germinated but dead after 3 days.

†Mean ± SE

Table 8-4. Growth of Fucus Zygotes and Laminaria Gametophytes After Treatment in Oil/Water Mixture During Gamete and Spore Release. Treatment was with No. 2 Fuel Oil.

Added	Total Extractable Hydrocarbons	Length (μm ± SE) of *Fucus* zygotes after 13 days growth	Diameter (μm ± SE) of *Laminaria* gametophytes after 21 days.
0	*	699 ± 21	164.4 ± 9.2
2ppb	*	0 ± 0	60.8 ± 1.2
20ppb	*	0 ± 0	**0 ± 0
200ppb	*	0 ± 0	0 ± 0
2ppm	(0-30ppb)	0 ± 0	0 ± 0
20ppm	(1-3ppm)	0 ± 0	0 ± 0
200ppm	(18-28ppm)	0 ± 0	0 ± 0

* Dissolved hydrocarbons undetectable by spectrophotometry due to extremely minute amounts Values are range of measurement and include all the petroleum products.

** Spores germinated but were dead after day nine.

investigators using different techniques and response parameters. Based on the bioassays with *Fucus* and *Laminaria*, it appears that they were not as sensitive to oil as some microalgae that have been studied (7), but they were similar or slightly more sensitive than bioassays developed with fish and invertebrates (2, 3). Although the toxicity values obtained in these studies were not directly applicable to *Fucus* and *Laminaria* (Pulich used doubling times and Eisler used LC_{50} values) similar values can be derived.

The extreme sensitivity of the reproductive stages, i.e., eggs and sperm in *Fucus*, and spores in *Laminaria*, indicate that either of these organisms might become a useful bioassay tool.

In one experiment, *Fucus* receptacles were allowed to stand in various concentrations of oil 5 hours before being placed in moist chambers overnight. After completing the experiment in the usual manner, and allowing gamete release to occur in sterile seawater, the deleterious effects on the sperm were not observed. However, growth of the juvenile plants was reduced, being similar to that in Table 8-2, even though the zygotes were not in oil solutions during or after fertilizations. Further experiments with two-week old juvenile *Fucus* plants indicated that as the plant gets older, sensitivity to oils decreases. Thus, the most critical stage of the life cycle, and the one most sensitive to oil, is the reproductive phase.

Continued development and refinement of these bioassay procedures is needed, as well as a survey of other seaweeds, to see how representative these results are for seaweeds in general. The development of a bioassay in a flow-through system would be more representative of natural conditions.

The deleterious effects of low-level oil pollution on the reproductive cycle of these algae can easily be visualized, especially in areas of chronic pollution, such as those found near harbors, marinas, and similar installations. By preventing the completion of the life cycle, the community structure of algae, as well as that of higher trophic levels, will be altered. Greater effort should be made to examine chronic, long-term effects of oil pollution on the marine ecosystem.

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EFFECTS OF NO. 2 HEATING OIL ON FILTRATION RATE OF BLUE MUSSELS, *MYTILUS EDULIS* LINNE

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ABSTRACT

Reductions in gill filtration rates were observed for adult blue mussels, *Mytilus edulis*, that were exposed in a continuous flow-through dosing system, to three concentrations of the water-accommodated fraction of No. 2 fuel oil. The oil concentrations were measured routinely by infrared spectrometry, and averaged 0.019 ppm, 0.06 ppm, and 0.64 ppm throughout the exposure period. Filtering rates for healthy, unexposed mussels ranged from 7.2 to 30.9 ml/min, depending on ambient water conditions. In comparison to controls, filtering rates decreased as the oil concentration increased, with significant reductions occurring at all dose levels within 48 hours of exposure. Continued oil exposure up to two weeks produced progressively higher reductions in filtering rate. When returned to uncontaminated water for two weeks, the mussels resumed their normal feeding rates, revealing that the effect was reversible. Mussels collected from a small oil spill site exhibited similar responses.

INTRODUCTION

Bivalve mollusks are of considerable value to ecologists studying the effects of pollution, because many of the species are sedentary filter feeders, and are likely to accumulate contaminants from their surroundings. *Mytilus edulis*, the blue mussel, has become one of the most widely studied members of the group since it has a worldwide distribution; it is easy to maintain in the laboratory; and it is exploited commercially, particularly in European countries. Also, *Mytilus*, because of its intertidal existence, is particularly vulnerable to oil exposure.

Several investigators have demonstrated a reduced feeding rate in mollusks exposed to environmental stress. Galtsoff *et al* (3) reported fifty percent reduction in gill ventilation rate of oysters exposed to an extract of crude oil.

They attributed this reduction to an anaesthetic effect upon the gill cilia. Preliminary investigations at the Environmental research Laboratory, Narragansett, revealed that feeding rates of clams, *Mercenaria mercenaria*, and scallops, *Argopecten irradians*, were notably diminished after exposure to No. 2 fuel oil (7).

A reduced feeding rate has also been noted for *Mytilus edulis*. For instance, Abel (1) observed reduced filtration rates in mussels exposed to various pollutants including copper, zinc, mercury, cyanide, thiocyanate, and sulfide. González and Yevich (5) reported that *Mytilus* show a significant decrease in filtering rate when exposed to high temperatures in the laboratory. Gilfillan (4), investigating the response of *Mytilus* to seawater extracts of crude oil, reported a decrease in both food consumption and assimilation, and an increase in respiration. The combined effects resulted in a reduction in the net carbon flux at oil concentration as low as 1 ppm.

The Oil Pollution Research Branch at the Narragansett Environmental Research Laboratory has been assigned the task of evaluating the effects of very low levels of oil on ecologically and commercially important marine species. Such levels may not immediately lead to death of the organisms, but may ultimateiy jeapordize their long-term success at survival. Since *Mytilus edulis* is an important species in the marine community, and since a change in filtration rate appears to be a well-defined response to environmental disruption, we conducted an investigation to elucidate the behavioral effects of very low levels of oil, and to evaluate the recovery potential of the stressed animals.

METHODS

Adult *Mytilus edulis* were collected from the southeastern shore of Conanicut Island, Rhode Island, in April, 1976. In the laboratory, the animals were measured and separated into four groups of 50 individuals each. Mean shell length of the mussels was 4.63 cm. Each group of mussels was maintained for a two-week acclimation period in a plastic coated wire cage that was suspended in a one meter diameter fiberglass tank. The tanks were supplied with continuously renewed unfiltered seawater, which allowed the mussels to feed on natural plankton from the incoming water.

After the acclimation period, the filtration rate was measured for each of the four groups of mussels. Next, these animals were placed for two weeks in a flow-through oil exposure system designed by Hyland *et al.* (6). One group was placed in each of three nominal oil concentrations–0.01 ppm, 0.1 ppm, and 1 ppm– and one group was held under control conditions. Filtration rates were measured at various intervals during the two-week oil exposure period. All animals were then transferred to control conditions, and their filtration rates measured periodically to determine recovery.

Gill filtration rate was measured indirectly by recording the rate at which the animals removed food particles from the surrounding water. Each cage of 50 mussels was transferred from its exposure or recovery tank to individual glass aquaria containing equal and known quantities of *Isochrysis galbana*. The algal suspension was maintained by aeration. Subsequently, three replicate 25 ml water samples per aquarium were withdrawn at intervals during a three-hour feeding period. The number of algal cells in each sample was counted on a Coulter electronic cell counter, and the average percent reduction through time recorded for each aquarium.

Filtration rates were expressed both graphically and numerically. Feeding curves were generated by plotting percent food particles removed versus time to allow visual comparison between the feeding rates of the control and oiled mussels for each of the various exposure or recovery periods. Results were analyzed statistically by performing linear regressions on the natural log transformed data, and comparing the regression lines (9). Actual filtration rate–the rate at which a solution is pumped through the gills of the animal in a given time period–was determined numerically with the aid of the following formula (8):

$$\text{Filtration rate (ml/min/mussel)} = \frac{\text{vol. solution (ml)}}{\text{(no. animals)} \times \Delta \text{T min)}} \times \ln \frac{C_o}{C_t}$$

where C_o and C_t represent food concentrations at the beginning and end of a particular feeding interval (ΔT). The solution is based on the assumption that if filtration rate remains constant over the feeding interval, then the rate at which particles are removed from suspension will decline exponentially, as described by the curve e^{-x}. For a given group of mussels, filtration rate was finally expressed as the average of those values calculated separately for each interval during which the mussels were actively removing food particles. Averaging is necessary to correct for the fact that the calculation of filtration rate can vary slightly depending on the magnitude of the time interval selected, a result of the fact that particles are not always removed at an exact exponential rate.

The flow-through oil exposure system is designed to dose marine animals with the water-accommodated fraction of No. 2 fuel oil at three nominal concentrations–0.01 ppm, 0.1 ppm, and 1 ppm. The W.A.F. contains finely dispersed oil as well as the water-soluble components, but does not include the whole oil slick. The system simulates an area of chronic petroleum hydrocarbon pollution, such as one that might exist near a sewage outfall, an oil refinery, or an area consisting of sediments that have been heavily contaminated with oil.

Temperature, salinity, dissolved oxygen, and pH were routinely measured in the dosing tanks, and averaged 16°C, 31 ppt, 8.09 ppm, and 8.03 respectively. Hydrocarbon concentration was also determined routinely by infrared spectrometry, following the techniques described in Hyland, *et al.* (6). The actual oil concentrations measured according to this method, are somewhat different from the nominal ones mentioned previously. Accordingly, during the exposure period the 0.01 ppm tank averaged 0.019 ppm above the natural background hydrocarbon concentration; the 0.1 ppm tank averaged 0.06 ppm; and the 1 ppm tank averaged 0.64 ppm.

RESULTS AND DISCUSSION

Figure 9-1 illustrates the feeding activity of *Mytilus edulis* prior to oil exposure. There appears to be little difference in the shapes of the four feeding curves; and, in fact, statistical analysis revealed no significant differences ($P < 0.05$). Typically, 80 percent of the food particles were removed by the mussels in 15 minutes, and 95 percent in 30-minutes, at which point maximum filtering activity was reached. Over this 30 minute interval, the average filtration rate for the four groups was calculated as approximately 18.1 ml/min, which is representative of values reported elsewhere in the literature (2). The values ranged from 15.6 for the control; to 18.7 for the 1 ppm group, and 19.1 for both the 0.01 and 0.1 ppm groups (Table 9-1).

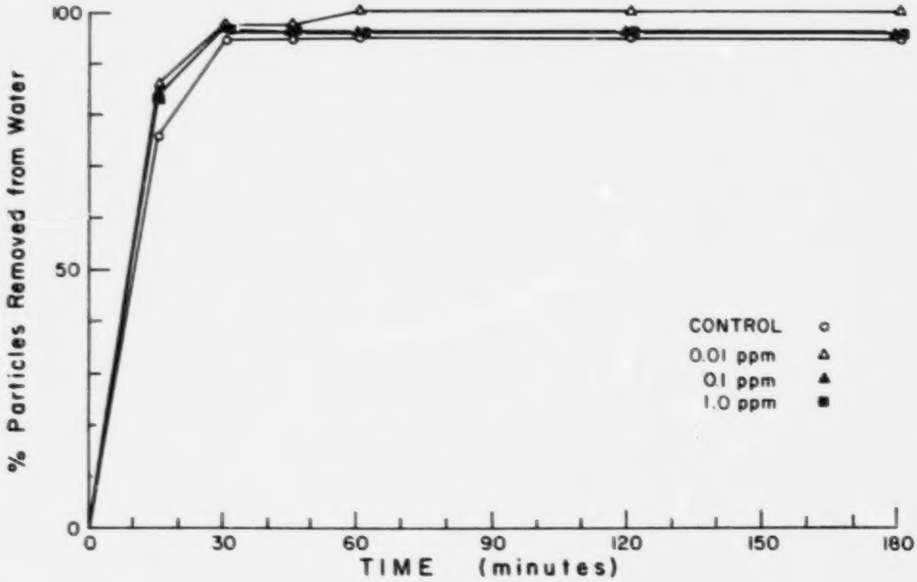

Figure 9-1. Pre-exposure: Comparison of Filtering Activity of *Mytilus edulis* Prior to Exposure to W.A.F. No. 2 Fuel Oil.

Table 9-1. Filtration Rates for Control and Oil-Exposed Mussels.

NOTE: Mean and standard error (in parentheses) are both given.

	Control	0.01 ppm	0.1 ppm	1.0 ppm
Pre-exposure	15.6	19.1	19.1	18.7
	(0.4)	(0.4)	(1.9)	(1.5)
48-hr. Exposure	19.3	10.5	5.2	2.2
	(3.3)	(0.9)	(0.5)	(0.2)
2-wk. Exposure	17.8	5.3	1.8	0.3
	(1.5)	(0.3)	(0.5)	(0.04)
24-hr. Recovery	15.8	9.9	3.9	0.8
	(0.2)	(1.2)	(0.2)	(0.1)
2-wk. Recovery	30.9	–	30.9	17.2
	(6.5)		(6.5)	(1.6)

After 48 hours of exposure (Figure 9-2), the filtration curves for the three experimental groups began to diverge, while the control curve retained pre-exposure characteristics. Mussel feeding activity in all three oil concentrations was significantly reduced from that of the control, with the highest concentration producing the most severe reduction. Filtration rates for the 0.01 ppm, 0.1 ppm, and 1 ppm polluted mussels, decreased to 10.5, 5.2 and 2.2 ml/min, respectively, while the control group filtered at an average rate of 19.3 ml/min. Figure 9-3 illustrates that continued oil exposure produces progressively lower filtration rates. Mussels exposed for two weeks to 0.01 ppm required two hours to filter what the controls filtered in 30 minutes. Similarly, after three hours, animals exposed to 1 ppm had only consumed approximately 35 percent of the algae; while the controls far surpassed this in less than 10 minutes. After two weeks of exposure, filtration rates for the three exposed groups decreased to 5.3, 1.8, and 0.3 ml/min, while the control group filtered at an average rate of 17.8 ml/min.

Following the two-week exposure period, the animals were returned to clean water. Some evidence of recovery was noted after 24 hours in clean water (Figure 9-4); however, the filtration curves for all three exposure groups were still significantly different from the control. Filtration rates increased to 9.9 ml/min for the 0.01 ppm group, 3.9 ml/min for the 0.1 ppm group, and 0.76 ml/min for the 1.0 ppm group.

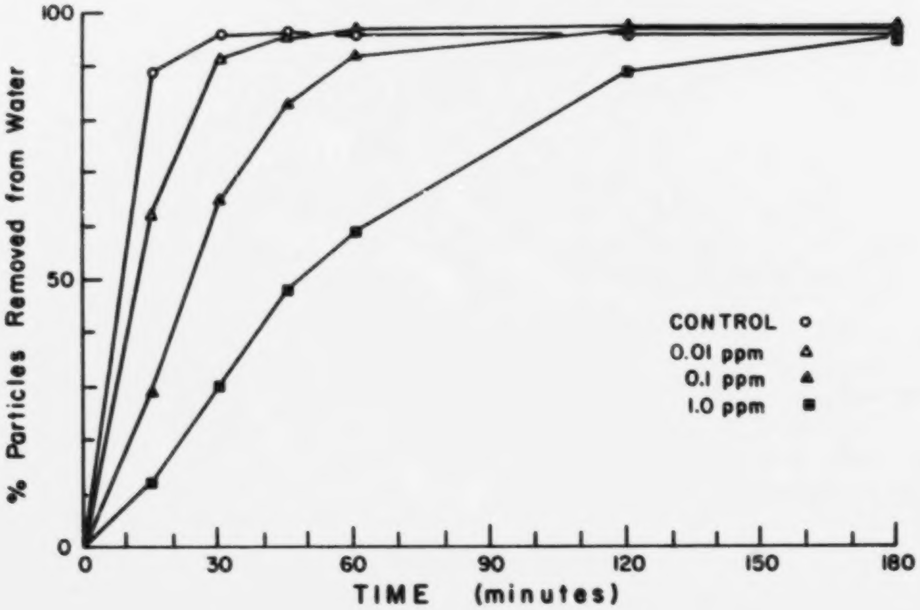

Figure 9-2. 48-hour Exposure: Comparison of Filtering Activity of Mytilus edulis Exposed to W.A.F. No. 2 Fuel Oil.

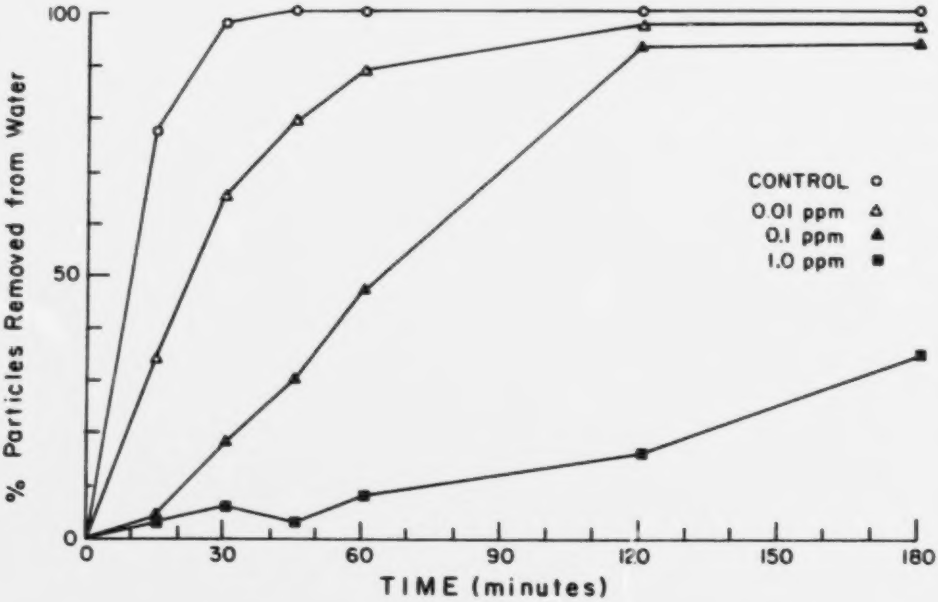

Figure 9-3. Two-week Exposure: Comparison of Filtering Activity of Mytilus edulis Exposed to W.A.F. No. 2 Fuel Oil.

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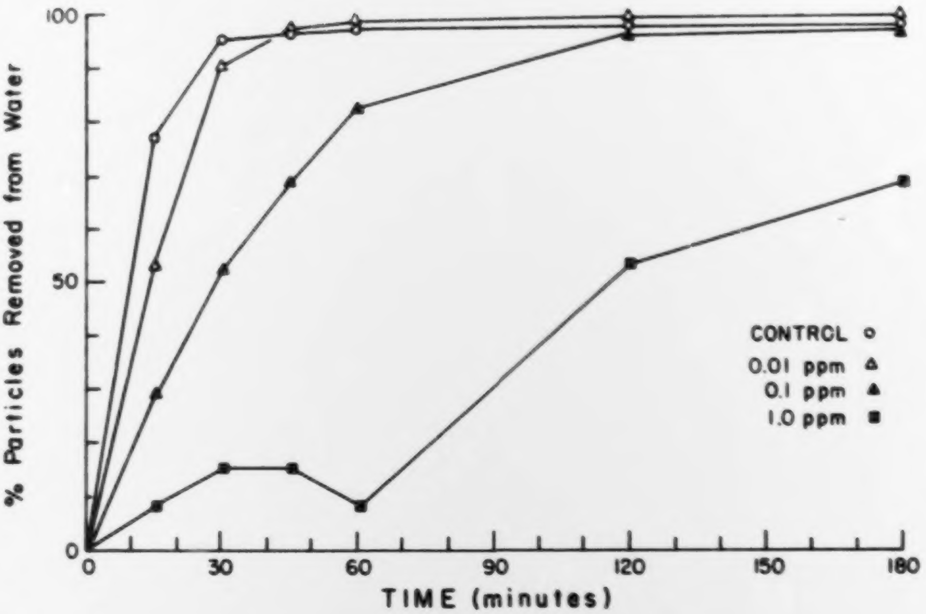

Figure 9-4. 24-Hour Recovery: Comparison of Filtering Activity of *Mytilus edulis* After 24 Hours of Recovery from Two Weeks Exposure to W.A.F. No. 2 Fuel Oil.

Gradual improvement of all groups was observed as the animals remained in clean water. After two weeks, recovery was almost complete (Figure 9-5). The control and 0.1 ppm groups both filtered at an accelerated rate of 30.9 ml/min, and the 1.0 ppm group filtered at a rate of 17.2 ml/min, characteristic of pre-exposure rates. The higher filtration rates observed may reflect increasing ambient water temperatures at the time of testing. Temperatures increased from 11°C at the time of pre-exposure testing, to 19°C during this latter testing period. Due to a laboratory failure resulting in reduced water flow, and subsequent anaerobic conditions in the recovery tank which held the 0.01 ppm exposure group, it was necessary to discard these mussels without demonstrating their complete recovery. However, since mussels at higher oil concentrations did recover, it seems reasonable to assume recovery for the 0.01 ppm exposure group as well. There remained a significant difference between the 1 ppm exposure group and the controls after two weeks in clean water; however, after one month of recovery, they actually fed slightly better than the controls.

Based on the current investigation, it appears that the adverse effect of oil on filtration rate of mussels is reversible, provided the stressed animals are returned to unpolluted conditions. However, the data also strongly suggest that recovery does not occur under conditions of continued exposure. Further investigation is currently in progress to determine the implications of reduced

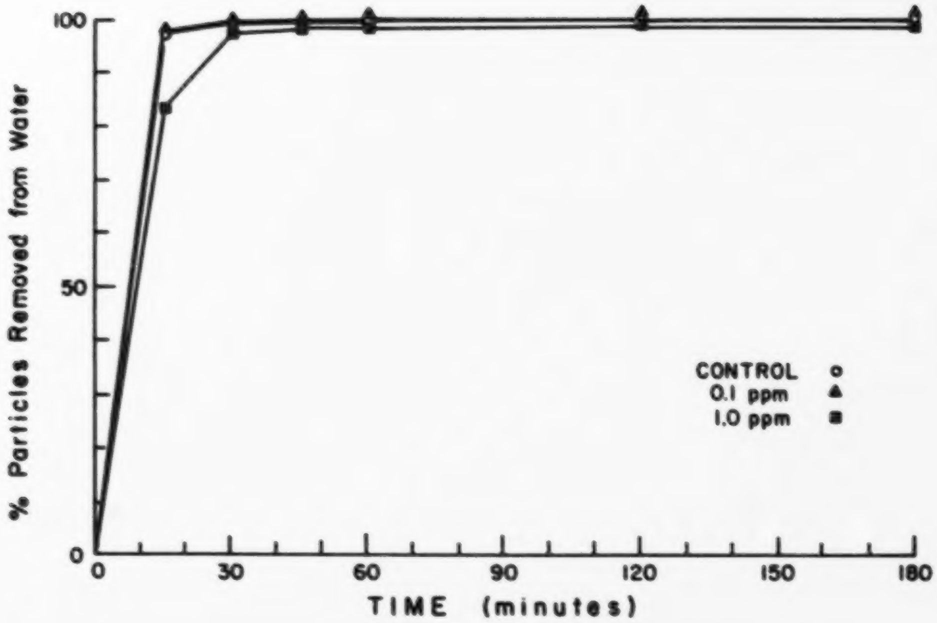

Figure 9-5. Two-week Recovery: Comparison of Filtering Activity of Mytilus edulis after Two Weeks of Recovery from Two Weeks Exposure to W.A.F. No. 2 Fuel Oil.

feeding over a long period of time as a result of continued oil exposure. A question confronted, for example, is whether mussels exposed for several months to chronic inputs of oil reveal reduced growth.

The laboratory experiments reported herein were designed to investigate responses to chronic oil pollution, and not the acute phenomena which occur immediately after an oil spill. However, in November, 1976, a small spill of No. 6 fuel oil occurred at Quonset Point, Rhode Island. The resulting slick drifted across the western passage of Narragansett Bay, and impacted approximately one mile of shoreline on Conanicut Island. This incident provided an opportunity to investigate the effects of spilled oil on filtering activity in mussels, and thus supports the laboratory results with field data. Accordingly, 48 hours after the spill, *Mytilus* were collected from the polluted site and from an unimpacted area nearby. Filtration rates were measured in the laboratory, and feeding curves were generated for both groups (Figure 9-6). Compared to controls, a small but statistically significant reduction in feeding activity was observed in oiled mussels. For example, over a period of 45 minutes, the controls had removed approximately 96 percent of the food particles, while the polluted mussels removed only 84 percent. Filtration rates were calculated as 7.2 ml/min (S.E. = 1.0) for the controls, and 4.9 ml/min (S.E. = 0.5) for the oiled group. The relatively low value obtained for the control group is most likely a reflection of low winter ambient water temperatures (5-6°C). One

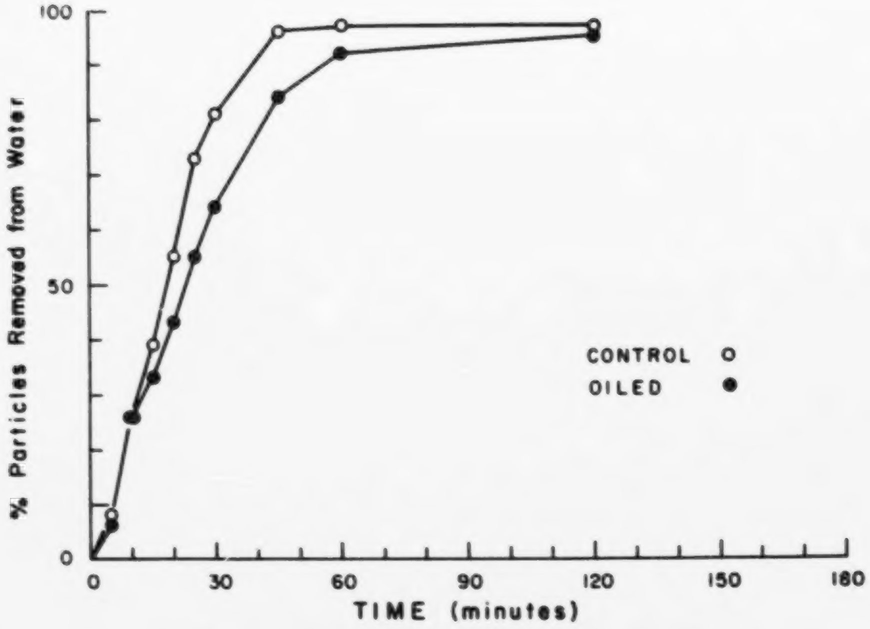

Figure 9-6. Oil Spill: Comparison of Filtering Activity of Mytilus edulis Collected From a Clean Area and from an Area Impacted by an Accidental Spill of No. 6 Fuel Oil.

week after the spill, another collection was made. Test results indicated that feeding activity of the oiled mussels had improved to the point that no differences could be found between control and oiled groups.

In conclusion, the investigation demonstrates that (1) under laboratory conditions an adverse reduction in filtration rate occurs in *Mytilus edulis* at very low levels of continuous oil exposure; (2) the effect is reversible, since recovery will gradually occur *if* the stressed animals are returned to unpolluted conditions; and (3) a similar effect occurs in response to spilled oil in the natural environment.

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LOBSTER BEHAVIOR AND CHEMORECEPTION: SUBLETHAL EFFECTS OF NO. 2 FUEL OIL

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ABSTRACT

Lobsters (*Homarus americanus*) were exposed in a flow-through oil dosing system to the water-accommodated fraction of #2 fuel oil. Behavioral observations of feeding efficiency and general behavior, showed that 5-day exposure to 0.08 and 0.15 ppm caused significant delays in feeding, without causing severe neuromuscular defects. Exposure to 1.5 ppm caused gross neuromuscular defects within 24 hours. Recovery was proportional to the gravity of observed defects. Neurophysiological experiments on antennular chemoreceptors of behaviorally observed animals showed that oil is perceived as a chemical stimulus, and can change normal responses to food juices. Oil-exposed animals show abnormal, bursting spike patterns, both spontaneously and in response to food juice. It remains to be proven that low level exposure effects are due to oil interference with chemoreception. This is a report on preliminary data.

INTRODUCTION

Each year more evidence appears which demonstrates the importance of chemical signals in the lives of marine animals. The following are just a few examples of the broad categories of behavior where chemical signals are of vital importance: feeding behavior, both the predator's detection of live prey and the scavenger's localization of dead bait; the prey's alarm and escape behavior; mating behavior and mate selection; parental brood recognition; and the selection of suitable geographic locations, as in larval settling and homestream return of migratory species. Interference with chemical signals or with the receptors that evolved to receive them could therefore jeopardize animal survival without causing immediately obvious deleterious effects on the individual. Man's chemical discharges into the environment, such as large amounts of petroleum hydrocarbons in coastal areas, may cause such interference.

Speculations about petroleum hydrocarbon interference with the processes of chemoreception have appeared with a certain regularity in the literature, starting with Blumer (4). The reasons for this speculation are obvious: petroleum hydrocarbons are a mixture of organic chemical compounds, some of which are related to compounds such as pheromones and alarm substances, which are utilized by animals for their orientation and communication. These communication signals may have chemical features, such as carbon skeleton, functional groups, volatility, and solubility, in common with compounds in petroleum (7). In an oil-polluted environment, different petroleum compounds will be in solution or in emulsion in the water column, while the heavier fraction can become part of the benthic mud and affect the benthic ecosystem for many years, as shown by Blumer and Sanders, among others (5, 9). One can thus envisage the scenario when these chemical look-alikes mimic or mask the reception of biologically important signals. Mimicked signals may result in "false alarm", i.e., animals may look for imaginary food or mates, or avoid predator danger where there is none. If their chemical signals are masked, animals cannot respond to them and may miss opportunities to feed, or mate, or escape. A third possibility less frequently mentioned is that animals may become subject to two competing signals (3), for instance, an attractant signal from food (or mate) and a repellent signal from oil. In such cases chemoreception would be perfectly normal, but the animal may not be able to decide whether to feed or hide. Such delays may be more critical than apparent at first glance: even a slight delay in responding to food can put an individual at a significant disadvantage when competing with an unimpaired conspecific, or in escape from predators.

Thus far, some cases of mimicked food attraction and delayed food responses have been observed, as well as increases and decreases in alarm and attraction behavior (1, 8, 10, 11). However, specific effects of oil on chemoreception itself have never been documented. Studies showing oil interference with chemoreception will provide us with a general understanding of the effects of oil pollution, since the processes of chemoreception – although essentially unknown – are probably similar in all animals. This would be especially true if similar effects for petroleum fractions were found in different animals. Interference with chemoreception or chemically mediated behavior also may be one of the most sensitive measures of low level oil pollution, since the much more obvious neuromuscular abnormalities appear at higher, although still sublethal, levels of oil exposure.

For this study we chose the bait localization behavior of the lobster, *Homarus americanus*. The lobster uses two chemoreceptor organs. Aesthetasc hairs on the antennules represent their sense of smell, and function probably to detect distant chemical signals in low concentration. Hairs on the walking legs and maxillipeds are the equivalent of taste, and are essential in picking up food and bringing it into the mouth while testing its palatability for ingestion. In this study, using the water-accommodated fraction (WAF) of #2 fuel oil, we are mainly concerned with distance chemoreception of the antennules.

The first purpose of these experiments is to determine the range of #2 fuel oil exposures that affect the feeding behavior of lobsters without causing neuromuscular disturbance. Since chemoreception provides an important input into their feeding behavior, we then apply neurophysiological techniques to measure the effects of oil exposure on chemoreceptors in animals, where sublethal behavioral abnormalities have been shown. This is the second goal of these experiments. The results reported here are preliminary; the methods are established.

MATERIALS AND METHODS

Flow-Through Oil Dosing System

In order to measure actual exposure levels, a continuous flow-through oil dosing system is necessary. The flow-through system (Figure 10-1) consisted of two head tanks, one control and one experimental. The experimental head tank, 8' x 11" x 8", was fitted with three baffles to aid in the layering of the oil after mixing. Its inflow was 4,000 ml/min. Oil was introduced via a syringe pump at a fixed rate into the center of the fast jet of seawater, causing rapid emulsification. The overflow of the head tank was skimmed off into a collecting tank where the oil layer was siphoned off occasionally. From the head tank, the oil-water mixture entered six 100-liter tanks individually.

The overflow from the individual tanks entered a holding tank where lobsters were stored for neurophysiological preparations on oil-exposed animals. The overflow from the collecting box and the holding tank entered an acrylic-fiber filter box, where oil was removed before the water entered the drain (Figure 10-1). The control head tank, 4' x 11" x 8", supplied four individual 100-liter tanks. Its inflow was 2,600 ml/min. Individual tanks, both experimental and control, had inflows ranging from 400-460 ml/min. Water quality – salinity, temperature, ammonia, pH and O_2 content – and flow rate to individual tanks were measured every other day.

Behavior

Two male and two female lobsters served as controls, three males and three females as experimentals. From our holding facility, we chose lobsters which had molted within two to eight weeks of the start of the experiment, to avoid effects of pre-molt behavior during observation. The animals were measured for close size match, and put in individual tanks containing a glazed clay shelter and a pebble substrate. They were fed twice daily until all animals were feeding normally. Then a base line for feeding behavior was determined over a five-day period. During the whole experiment lobsters were observed daily in the early morning (7-9 am) and late afternoon (4-6 pm). One-minute behavior recordings were followed by the addition of food, which was lowered on a string from the right or left front corners, alternately. Apart from general behavior (about 25

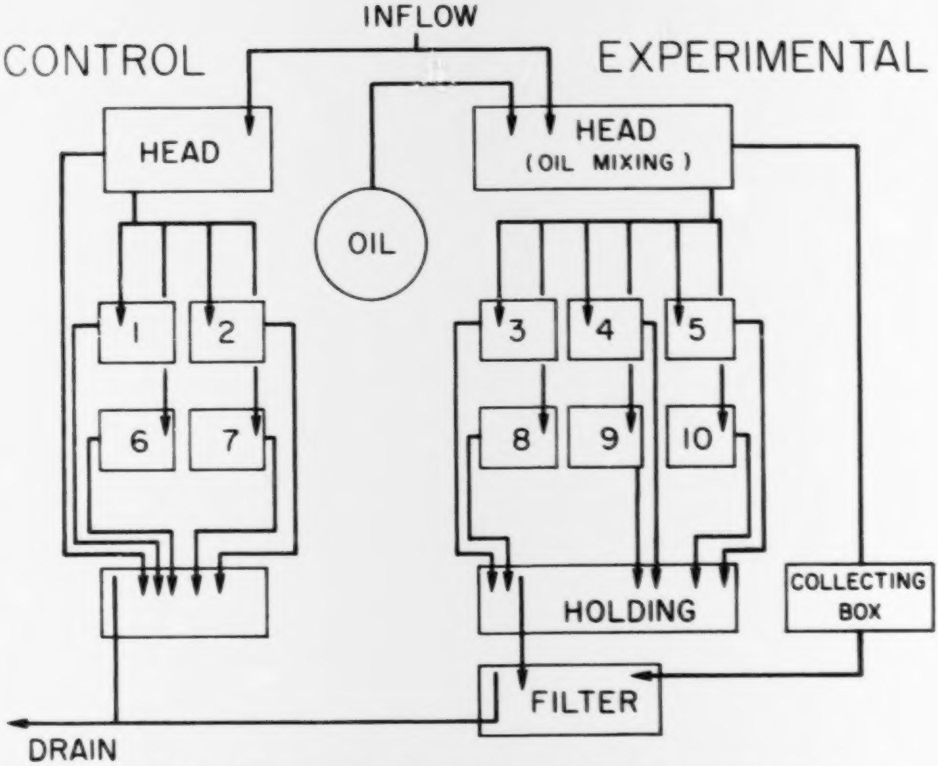

Figure 10-1. Diagram of Flow-through Oil Dosing System.
NOTE: See text for details.

different postures and movements) the times for alert (first observable response to food), wait (period between alert and leaving shelter to search), and search (time after first leaving shelter until food touched with maxillipeds) were recorded. On the morning of the 6th day, #2 fuel oil was introduced at a predetermined flow rate. Lobster behavior was recorded 6 hours later and subsequently twice daily, as above. After five days, oil introduction was stopped. All recordings were continued as before for another five days to determine behavioral and chemical recovery rates.

Oil Chemistry

Concentrations of total hydrocarbons in the water column were determined by infrared spectroscopy before, during, and after introduction of oil. On the day before oil was added, a 2-1 water sample from every tank was extracted with 50 ml CCl_4. A second extraction was performed 12 hours and a third 24 hours after onset of oil mixing. Extractions were continued daily for the remaining four days of oil exposure, and on the first and second day post-exposure to determine how quickly oil left our system. A small number of CH_2Cl_2 extractions were performed for gas chromatographic analysis. The oil used in the study was an Exxon #2, provided by the EPA Environmental Research Laboratory, Narragansett, Rhode Island.

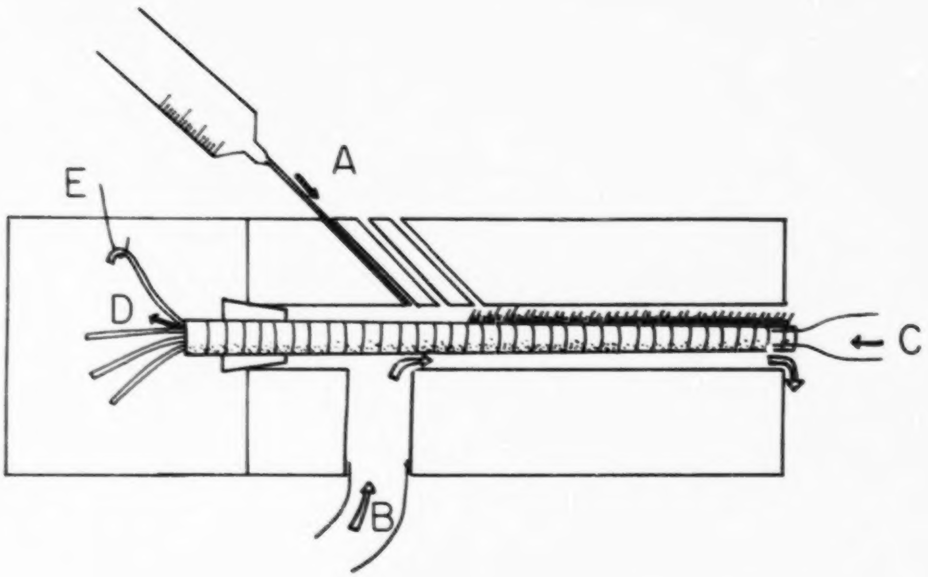

Figure 10-2. Diagram of Stimulation-Recording Chamber ("Olfactometer").

NOTE: Stimulus is injected with a syringe (A) into a seawater flow (B) over the dissected lobster antennule. The antennule is perfused through a micropipette with oxygenated lobster saline (C) which exits the antennule into a bath of saline (D). To make recordings, one small bundle of nerve fibers is lifted from the saline bath into the air with a platinum hook electrode (E). Seawater and saline baths are separated by a Sylgard cork, through which the antennule passes.

Neurophysiology

Electrophysiological data were obtained from chemoreceptors of oil-exposed and normal lobsters, some of which had been observed behaviorally. This permits a comparison between the neural chemosensory input the animal received, and the resultant behavior after processing through higher nerve centers. Such a comparison is a necessary step in determining whether oil interferes with behavior through chemoreception.

To measure neurophysiological activity, the lateral flagellum of the antennule of a lobster was removed and placed in fresh seawater. The cut proximal end was inserted through a Sylgard cork. Three to four cuticular rings were removed. The distal tip was cut and the antennule placed in a lobster saline bath in the stimulation-recording chamber (Figure 10-2). A micropipette was inserted snugly into the distal tip, and perfusion with oxygenated lobster saline started within 10 minutes after the removal of the antennule from the animal. Test chemical stimuli were injected into the continuously flowing saline bathing the antennule. Recordings were made by picking up a small nerve bundle with a platinum electrode. The signal was amplified via a Tektronix Type 122 Preamplifier, and displayed on conventional recording equipment for later analysis.

It is commonly accepted that neurophysiologically determined thresholds of sensory receptors lie an order of magnitude above the behaviorally determined thresholds. Thus, to document the effects of #2 fuel oil on lobster antennular chemoreception, we used the following test series: (1) mussel juice; (2) #2 fuel oil, 10 ppm: (3) mussel juice plus oil; (4) artificial seawater; and (5) mussel juice. Stock solutions were made at one time and refrigerated. Artificial seawater was made according to the MBL formula: 420 mM $NaCl$, 9 mM KCl, 9 mM $CaCl_2 \cdot 2H_2O$, 23 mM $MgCl_2 \cdot 6H_2O$, 26 mM $MgSO_4 \cdot 7H_2O$, 2 mM $NaHCO_3$ (pH 7.3). This was used to eliminate introduction of day-to-day variations in natural seawater. Mussel juice was made by homogenizing 10 g wet weight of *Mytilus edulis* tissue in 100 ml artificial seawater. The suspension was centrifuged at 27,000xg for 20 minutes and the pellet discarded. The supernate was frozen in small aliquots until needed. The WAF of 10 ppm #2 fuel oil was made at the start of each preparation, due to the lability of the oil-water suspension.

This protocol allowed us to compare the response to Stimulus (1) with the response to Stimulus (5) for nerve fiber damage or fatigue, or lasting effects of the prior oil test stimulus. Stimulus (2) and Stimulus (3) were used to determine a) whether lobster antennules can detect oil as a chemical stimulus, and b) if the presence of oil changes the response to mussel juice. Stimulus (4) was used to determine the sensitivity of the preparation to a chemically neutral stimulus; this allowed us to measure mechanoreceptor activity which can be subtracted to discover purely chemosensory responses in the other tests.

RESULTS

Chemistry

Water quality measurements showed that for all experiments, salinity and pH remained constant, ammonia remained undetectably low, and O_2 remained at saturation. Temperatures fluctuated with ambient water temperature, and are listed below with each experiment.

Gas chromatography showed that the water-accommodated fraction recovered from the lobster tanks closely resembled whole #2 fuel oil. Infrared spectroscopy of CCL_4-extractable lipids showed moderate (±20%) daily fluctuations within and between individual tanks. The dosing system was capable of maintaining relatively similar exposure levels.

Behavior

In the first experiment, the recovered oil level in the exposure tanks was about 0.08 ppm total hydrocarbon. The temperature gradually rose from 22° to 24.5° C over the 15-day experimental period. Behavioral changes were observed in the morning alert times, which, when comparing oil-exposed days with pre-exposure days, slowed in experimental animals ($p < 0.05$). Total food localization time was also slower ($p < 0.025$), perhaps as a result of slower alerting. Control animal behavior did not change (Table 10-1). In this first experiment, defensive postures and sometimes erratic and frantic behavior was observed in most of the exposed lobsters, and not in control animals. Defensive postures are characterized by wide open seizer claws, held close to the body, while the animal sits retreated far into its shelter. Erratic and frantic movements are sudden, unprovoked seizer snapping, jerky body movements and twitches.

In the second experiment, the recovered oil level was about 0.15 ppm; temperature was a constant 10° C. Neither oil-exposed nor control animals showed significant changes in feeding behavior in the morning obervation, when comparing pre-oil with oil exposure period. In the afternoon observation, oil-exposed animals did not change their search speed but their alert was delayed ($p < 0.05$). Control animals had a faster search time in the afternoon ($p < 0.005$). Both control ($p < 0.01$) and experimental animals ($p < 0.05$) showed shorter wait times (Table 10-1).

In the third experiment the temperature rose from 11° to 13.5° C, and the recovered oil level was 1.5 ppm. At 30 hours the lobsters showed gross neuromuscular defects, and oil inflow was stopped. In this experiment, behavior in post-exposure recovery period was compared with pre-exposure behavior. Experimental lobsters were slower in all phases of feeding behavior during the five-day recovery period than in the five-day pre-oil period ($p < 0.001$). Even five days after exposure to 1.5 ppm #2 fuel oil for 30 hours, half the lobsters did not feed within the 10-minute limit (Figure 10-3). Control lobsters showed no significant differences (Table 10-1). The animals during this last experiment showed three levels of effects, some animals being affected much more than others. A description of the three levels follows (see also Figure 10-3).

- *Most extreme* (two lobsters) –After 30 hours of oil exposure, these lobsters were found outside their burrows lying on their backs, pleopods twitching or still, tail half curled, walking legs twitching, antennae and antennules limp, gill bailers moving slightly. Occasionally the back and tail were arched and then curled. At times, attempts were made to right itself. Body jerked after food entered into tank. No recovery occurred in five days.

Table 10-1. Significant Changes in Feeding Behavior of Lobsters Before and During Exposure to #2 Fuel Oil (WAF)*.

Level of Exposure	0.08 ppm				0.15 ppm				1.5 ppm			
Observation Time	AM		PM		AM		PM		AM		PM	
	Cont	Exp	Cont	Exp	Cont	Exp	Cont	Exp	Cont	Exp	Cont	Exp
Alert	=	s(0.05)	=	=	=	=	=	s(0.05)	=	s(0.001)	=	=
Wait	=	=	=	=	=	=	f(0.01)	f(0.05)	=	s(0.001)	=	s(0.001)
Search	=	=	=	=	=	=	f(0.005)	=	=	s(0.025)	=	s(0.001)
Total	=	s(0.025)	=	f(0.05)	=	=	=	=	=	s(0.01)	=	s(0.001)

* In 1.5 ppm exposure significant differences are listed for the pre-exposure period compared with the post-exposure period; the other two experiments list differences between pre-exposure and exposure periods.

NOTE: Measured times were equal (=), slower (s), or faster (f) than in the pre-exposure control period. Significance levels are given in parentheses.

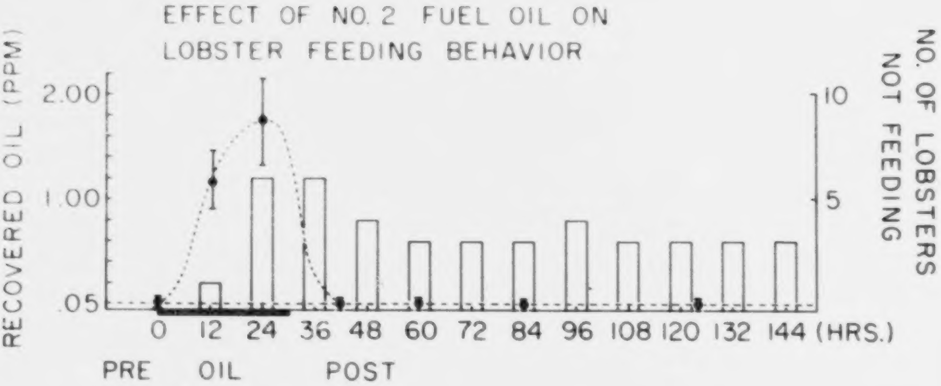

Figure 10-3. Effect of 1.5 ppm No. 2 Fuel Oil (WAF) on Lobster Feeding Behavior.

NOTE: Large bars indicate number of lobsters not feeding within 10 minutes of food introduction. Broken line represents estimated oil levels in lobster tanks based on actual recovery measurements (indicated by dots and standard deviation bars). Hatched horizontal bar shows period of oil introduction into the system. "Pre" is the 5 day pre-exposure period: all lobsters feed and recovered oil remains below 0.05 ppm. "Oil" is the exposure period: highest mean exposure level 1.7 ppm, oil stopped after 30 hrs. "Post" is the recovery period: oil quickly leaves the system, feeding behavior remains seriously affected for the entire 5-day period.

- *Moderate* (three lobsters) – These animals were generally out of their burrows in two alternating stances. In one, the tail and head were down, the antennae were folded back, the antennules beat slowly, and the animal lay down low on the walking legs. This appeared to be a resting position. Then the tail and head arched up, the claws were opened and held close to the body, the antennae and antennules were held straight up and together, animal stood high on walking legs, and made frequent tail flips. Poor coordination was exhibited. Frequent aggressive lunges were made with open, raised seizer claw, or jabs with both claws at no obvious target. Lobsters were unaware of the presence of food, at times walking right over it without responding. If they did pick up the food, they continued to wander aimlessly around the tank with the food clutched tightly in the maxillipeds. Recovery in five days was almost complete in two animals; one animal did not recover.

- *Light* (one lobster) – This animal remained in its burrow but was high on its walking legs, "spider" position and shaking. There were sporadic alerts not necessarily related to food. It exhibited no search. After two days recovery, there were slow hesitant approaches to the food with tail flips at the slightest irregularity. At the end of five days there was complete recovery.

Neurophysiology

The preliminary results from neurophysiological experiments on the lobster's olfactory chemoreceptors are presented with a few examples in Figure 10-4. Details are provided in the figure legend. These examples show that (1) the water-accommodated fraction of #2 fuel oil itself can be perceived as a stimulus by the primary receptor cells, (2) that the presence of oil in a mussel juice food stimulus can change the response pattern of the small nerve bundle, and (3) that exposure to oil causes abnormal bursting patterns (See Figure 10-4).

Generally, differences in chemoreceptor responses between mussel and mussel-plus-oil are more distinct in oil-exposed lobsters than in controls. One other striking feature is the tendency of oil-exposed individuals to exhibit irregular bursts, or frequent small clusters, of spikes, both spontaneously and in response to stimuli (Figure 10-4), This may be a general injury response (6) here caused by oil exposure. However, it has also appeared in nerves from animals which were exposed to very low levels of oil (0.3 ppm) in response to mussel-plus-oil stimuli, but not to mussel alone.

DISCUSSION

Our experiments have shown thus far that the original hypothesis that oil pollution may interfere in a number of different ways with chemoreception, and hence marine animal behavior, is not unreasonable. Behavioral experiments on the efficiency of the lobster's chemically mediated feeding behavior have shown that exposure to #2 fuel oil (WAF) causes significant delays after five days at exposure levels as low as 0.08 and 0.15 ppm. Increased dosage caused increasingly severe effects. Also behavioral recovery was a function of exposure level. At higher exposures (1.5 ppm) serious neuromuscular abnormalities appeared within 30 hours. Lobsters showed great individual differences in behavioral effects and recovery. The range of exposure levels where behavior was affected, but no serious neuromuscular defects appeared, proved to be surprisingly narrow. However, further experiments are required for complete documentation of this point.

Parallel neurophysiological experiments on the effects of such exposures on chemoreceptor performance showed that the receptors perceive oil as a chemical stimulus, that the presence of oil could modify normal responses, and that oil-exposed lobsters ofen showed abnormal receptor activity, both spontaneously and in response to food stimuli.

Based on these results, it appears that #2 fuel oil (WAF) interferes with lobster behavior in a number of ways. At low exposure levels (0.1 ppm range),

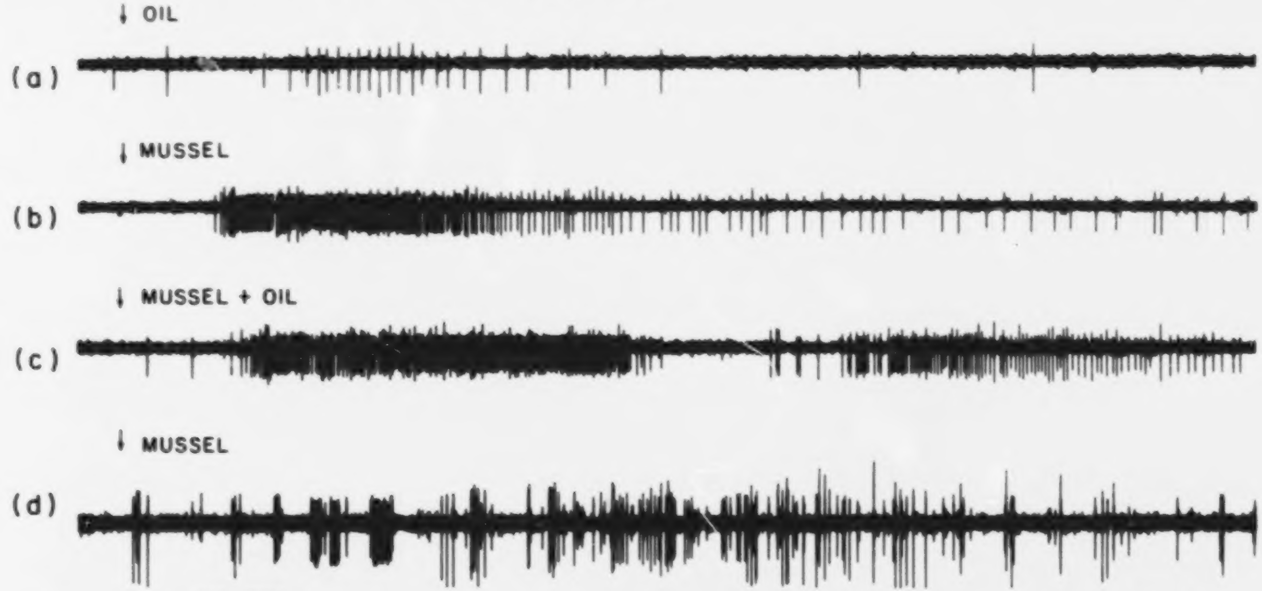

Figure 10-4. Examples of Neurophysiological Responses of Antennular Chemoreceptors to Chemical Stimuli.

NOTES:

a. Control lobster, response to oil stimulus.

b. Control lobster, response to mussel juice stimulus.

c. Control lobster, response to mussel juice-plus-oil stimulus.

d. Oil-exposed lobster, response to mussel juice stimulus. Note the differences between b and c which were recorded from the same nerve bundle a few minutes apart. Apparently the addition of oil changes the response to mussel juice. Note also the differences between b and d. The irregular bursting patterns seen in d are observed in oil-exposed lobsters, not in control lobsters. While there still is a response to mussel juice, the response pattern appears erratic. It is not known if this erratic pattern results in a different interpretation by the lobster.

the observed effects on behavior may be due to oil-induced changes in chemoreception. At higher exposure levels (1 ppm range), the behavioral effects appear neuromuscular, with a loss of coordination and equilibrium.

These preliminary results are being corroborated at different exposure levels. In additional experiments, we will attempt to provide a correlation between behavioral and neurophysiological results of oil exposure to determine if the observed behavioral effects of oil are caused by a malfunctioning chemoreceptor system.

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INFLUENCE OF NO. 2 FUEL OIL ON SURVIVAL AND REPRODUCTION OF FOUR MARINE INVERTEBRATES

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ABSTRACT

Responses to the water accommodated fraction of No. 2 fuel oil were determined in three marine gastropods (*Nassarius obsoletus*, *Crepidula fornicata* and *Urosalpinx cinerea*), and one Crustacean (*Cancer irroratus*). Experiments were conducted in either flowing or static systems at the following nominal oil concentrations: 0.0 ppm (control), 0.01 ppm, 0.1 ppm, 1.0 ppm. Mortality of adults and larvae was consistently pronounced only at a nominal concentration of 1.0 ppm. Toxicity to adult *N. obsoletus* at this concentration was greater during the winter than during the summer. Presence of sediment accelerated mortality during the summer, but had no effect on winter mortality. Exposure of adult *N. obsoletus* and *U. cinerea* to oil concentrations as low as 0.01 ppm and 0.1 ppm, respectively, interferred with normal patterns of egg capsule deposition. Exposure to oil did not alter the number of eggs/capsule in *N. obsoletus* or *U. cinerea*, and embryos produced by oil-exposed snails were viable. Fecundity of *N. obsoletus* may be reduced at a nominal concentration of 0.10 ppm. Growth rates of larval *N. obsoletus* and *C. fornicata* were reduced at nominal levels of 0.01 ppm and greater. Larvae of *C. irroratus* reared at a nominal concentration of 0.1 ppm weighed less at all zoeal stages relative to controls, even though carapace length of each larval stage, and time required to reach the megalops stage of development, were not altered.

INTRODUCTION

Lethal effects of petroleum hydrocarbons have been documented for a variety of marine organisms (21), including zooplankton (20) and both adult and developmental stages of benthic invertebrates (2, 9, 11, 19). Sublethal concentrations of hydrocarbons are also known to interfere with aspects of invertebrate reproduction, such as sperm motility and fertilization success (22, 25), and embryonic cleavage rates (1, 22). Reduced egg production has been reported for oil-exposed *Mytilus edulis* (7) and *Eurytemora affinis* (5), and development of some larval crustacea (16, 32, 33) and bivalves (9, 24, 25) is delayed after exposure to sublethal oil concentrations. Oil-induced changes in larval behavior of *Homarus americanus* (32) and *Cancer irroratus* (6) have also been demonstrated. Recently, Anderson *et al* (3) have reported effects of low hydrocarbon levels on hatchability of fish embryos and on heart beat rate of larval fish.

The general objective of this investigation was to elucidate some sublethal effects of No. 2 fuel oil (introduced as the water accommodated fraction, WAF) on aspects of the reproductive and developmental biology of several common coastal invertebrates. Specific topics studied include egg capsule deposition, fecundity, hatchability and larval growth rates. The lethal dose of the oil was also determined for adult and larval *Nassarius obsoletus*, and larvae of *Crepidula fornicata* and *Cancer irroratus*, in order to establish sublethal exposure levels.

A review by Moore and Dwyer (21) suggest that larval organisms are more sensitive to hydrocarbon toxicity than are adults, yet tolerance data on adults and larvae of the same species are infrequently reported. Culliney *et al* (12) suggest that the high surface/volume ratio in larvae and their "obligatory exposure to whatever may be in the water" would make larvae particularly susceptible to toxic substances, such as oil, at very low concentrations. Our study includes work on both adults and larvae of *N. obsoletus* to further examine this hypothesis.

MATERIALS AND METHODS

Experiments were conducted using the gastropods *Nassarius obsoletus*, *Crepidula fornicata* and *Urosalpinx cinerea*, and the crustacean *Cancer irroratus*. Adults were exposed to oil using the flow-through oil-dosing system described by Hyland *et al* (15). Briefly, unfiltered seawater and No. 2 fuel oil enter a mixing chamber. The WAF produced is then metered into exposure tanks where it is diluted to the desired concentration by controlled flow of untreated seawater. Total hydrocarbon concentrations in control (for background) and experimental tanks are monitored three times/week by infrared spectrophotometry, and flow rates are adjusted to maintain desired WAF exposure levels. Nominal total hydrocarbon concentrations (WAF) were: 0.0 ppm (control), 0.01 ppm, 0.1 ppm, and 1.0 ppm. Because measured hydrocarbon concentrations varied with time in the flow-through system, the nominal concentrations (cited as "X" ppm in the text) indicate only the order of magnitude dose level employed. Mean total hydrocarbon concentrations (± s.d.) measured during each experiment are given with the results; between treatment mean hydrocarbon values were significantly different for all experiments ($P < 0.05$).

All three gastropod species studied produce egg capsules. While *C. fornicata* broods its capsules, *U. cinerea* and *N. obsoletus* attach capsules to solid substrates and then abandon them, making these capsules easy to collect and count. Descriptions of larval development have been published for *N. obsoletus* (29), *C. fornicata* (34) and *C. irroratus* (28).

Adult Mud Snail Survivorship and Egg Capsule Deposition

Experiments with adult mud snails were conducted in the flow-through dosing system described above. Adults of *N. obsoletus* were collected from Bissell Cove, Rhode Island, and groups of 35-100 individuals placed in circular plastic containers (26 cm diameter, 6 cm high) and completely submerged in the dosing tanks. The top and sides were perforated to permit water circulation. Surface area of the top and side of each container was approximately equal. Snails were fed shredded *Mercenaria mercenaria* tissue weekly, and the number and position of deposited egg capsules were recorded before capsules were removed each week. All container surfaces were wiped clean after each examination. Dead snails were counted and removed periodically. The mean number of eggs per capsule was determined for *N. obsoletus* in all treatments. Since *N. obosoletus* is primarily a deposit feeder (30), and sediments are known to accumulate petroleum hydrocarbons from seawater (15, 17), one experiment was run with mud added to evaluate its influence on toxicity.

Reproduction of Urosalpinx cinerea

Specimens of *U. cinera* were collected at Jamestown, R.I., in May, 1976, and groups of ten individuals were placed in perforated plastic freezer containers. Three boxes were submerged in the flow-through system at each of the following nominal oil levels: control (0.0 ppm), 0.01 ppm, 0.1 ppm. Freshly collected barnacles were provided weekly as food. Once each week, deposited egg capsules were counted and then removed. In July, a sample of egg capsules was taken from each treatment level to determine the mean number of eggs encapsulated. The number of females present in each container was determined in the middle of the experiment using the live-sexing technique of Hargis (13).

Larval Survival and Growth

Survival of larvae exposed to No. 2 fuel oil (WAF) was assessed primarily under static conditions. Glass scintillation vials were completely filled with water siphoned from the flow-through dosing tanks. Fifteen to 20 two to three day old *N. obsoletus* or *C. fornicata* larvae were pipetted into each vial, and *Isochrysis galbana* was provided as food at an initial density of about 1 x 10^5 cells/ml. Vials were then tightly capped to minimize volatilization of hydrocarbons. Each experiment was conducted in triplicate. Larvae were counted daily and survivors were transferred to fresh medium. The experiments were conducted at room temperature (21-23°C), well within the range for good larval growth (10, 31).

One preliminary flow-through experiment was conducted with about 100 two-day old *C. fornicata* larvae (approximately 420 µm in shell length), using a system similar to that described by Calabrese and Rhodes (10). Water from the oil-dosing tanks was siphoned at approximately 50 ml/minute into flow-through chambers containing larvae, and 200 ml *I. galbana* suspension added at the beginning and end of each day as a feeding supplement.

Completely filled, capped quart glass jars were used to determine the effects of the oil on growth and survival of larval *C. irroratus*. Seventy-five Stage I zoea were added to each jar. Larvae were fed *Artemia salina* nauplii provided in excess numbers. Larval mortality was determined daily, and survivors were transferred to fresh medium. These experiments were conducted at 15°C, the optimal temperature for development of *C. irroratus* (28), and under a 12L:12D photoperiod. In a separate series of experiments, at least five individuals of each larval stage were harvested from control and "0.1" ppm levels to monitor growth. Larval carapace lengths were measured using an ocular micrometer. Dry weights were then determined using a Perkin-Elmer electrobalance after the larvae were rinsed with distilled water and dried at 80°C for 24 hours in pre-weighed foil pans.

RESULTS

Adult and Larval Survival

Exposure to a nominal concentration of 1.0 ppm was found to be lethal for all adults and larvae tested. Concentrations of "0.1" ppm and "0.01" ppm were sublethal to all test organisms for the particular exposure periods of our experiments.

Mortality of *N. obsoletus* adults did not exceed five percent in any experiment at control, "0.01" ppm or "0.1" ppm exposure levels. Substantial mortality occurred only at "1.0" ppm. Pronounced seasonal variation in toxicity was evident (Figure 11-1). In one winter exposure, mortality at "1.0" ppm reached 50 percent within approximately 30 days, with the remaining snails dying during the subsequent 30 days. Similar results had been obtained in a preliminary experiment initiated the preceding February. A very different mortality profile was seen at "1.0" ppm in two summer exposures. In 1976, approximately 40 percent of the snails were still living at the end of three months (Figure 11-1). In 1977, presence or absence of sediment in the holding containers was added as another variable. While summer toxicity of the WAF was still relatively low, mortalities were substantially increased by the presence of mud (Figure 11-2). In contrast, the mortality pattern was unaffected by sediment in the winter.

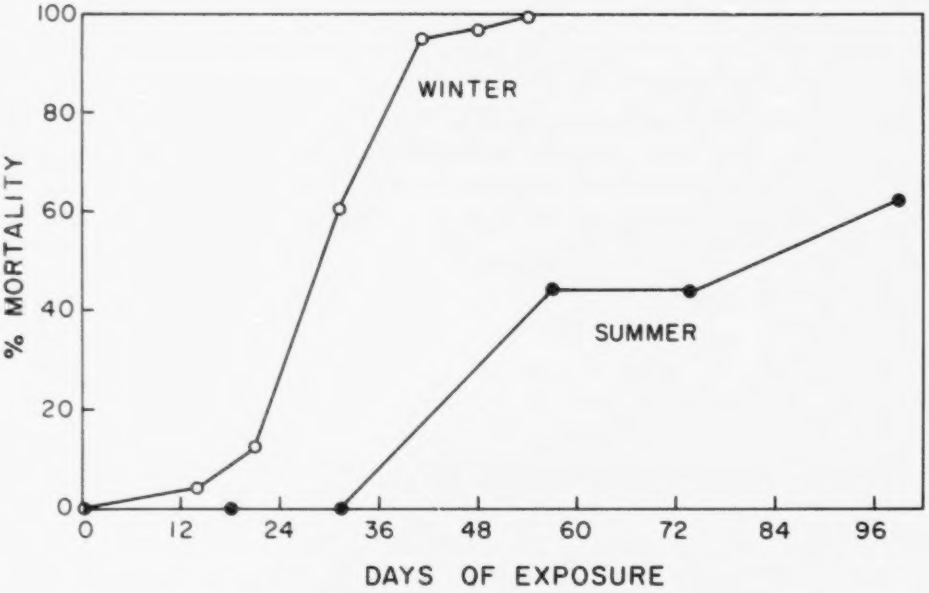

Figure 11-1. Survival of adult N. obsoletus exposed to "1.0" ppm No. 2 fuel oil (WAF).

NOTE: Control mortalities were less than five percent. Winter experiment was run 10/22/76 - 12/15/76 (100 snails/treatment). Summer experiment was run 7/6/76 - 10/12/76 (20 snails/treatment). Mean oil hydrocarbon concentrations ± s.d. (N) measured were: 1.25 ppm ±0.34 (25), winter; 0.94 ppm ±0.44 (31) summer.

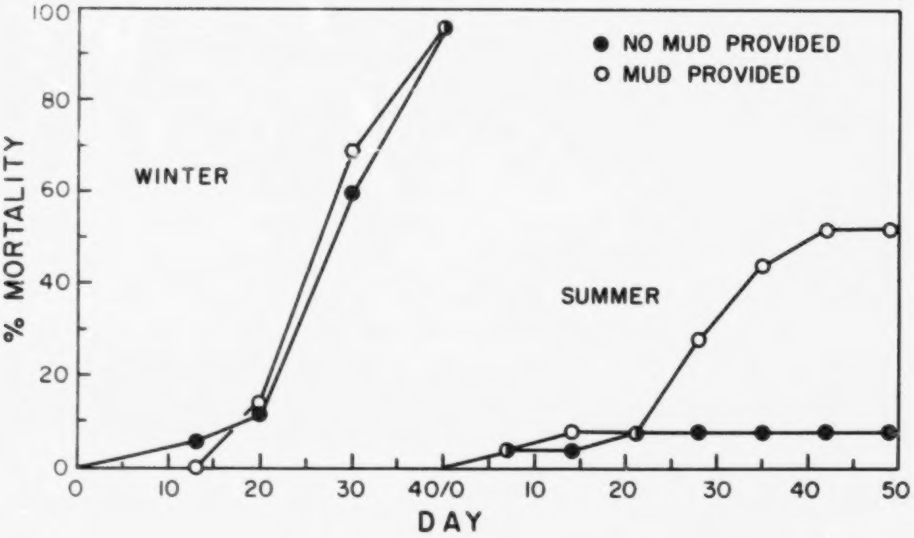

Figure 11-2. Survival of adult N. obsoletus exposed to "1.0" ppm in the presence or absence of sediment.

NOTE: Winter exposures were initiated 10/22/76 (50 snails/treatment). Summer exposures were initiated 7/12/77 (25 snails/treatment). Control mortalities less than 5 percent. Mean oil hydrocarbon concentrations ± s.d. (N) were: 1.25 ppm ±0.34 (25), winter; 0.70 ppm ±0.27 (23), summer.

Larval mortality of *N. obsoletus* was high in four to eight day experiments at "1.0" ppm, and relatively low at lesser concentrations (Table 11-1). Substantial batch variability in larval tolerance was observed (Figure 11-3). Whereas 50 percent mortality was recorded at "1.0" ppm after three-days in experiment "A", the 50 percent level was not exceeded until day eight in a second experiment using a different hatch of larvae. Indeed, no mortality occurred until day four in experiment "B".

All *C. irroratus* larvae exposed to "1.0" ppm died within four days. At "0.1" ppm and "0.01" ppm, about 33 percent of the larvae were still living after three weeks (Figure 11-4). Survival to the megalops stage, attained after 25-28 days, was: control, 58%; "0.01" ppm, 34%; "0.1" ppm, 30%; "1.0" ppm, 0%.

Table 11-1. Larval Mortality of N. obsoletus after Exposure to No. 2 Fuel Oil (WAF).

	Nominal Oil Concentrations			
	Control	0.01 ppm	0.1 ppm	1.0 ppm
Experiment A (4 days)				
Oil concentration (ppm)	0.0	0.0085 ± 0.0055(2)	0.093 ± 0.017(3)	0.98 ± 0.28(3)
Mean mortality (%)	11.6	0.0	5.9	81.6
Range of mortality (%)	0.0-20.0	–	0.0-8.3	53.6-100.0
Experiment B (8 days)				
Oil concentration (ppm)	0.0	0.012 ± 0.0089(4)	0.11 ± 0.024(4)	0.83 ± 0.19(4)
Mean mortality (%)	5.9	26.1	13.7	65.5
Range of mortality (%)	0.0-10.5	12.5-40.0	6.7-23.5	55.0-83.3

NOTE: Experiment "A" employed 15 larvae/vial and ran 4 days. Experiment "B" employed 20 larvae/vial and ran 8 days. The range of mortality observed in the 3 replicates run at each concentration is indicated. Oil concentrations are reported as mean ppm ± s.d. (N).

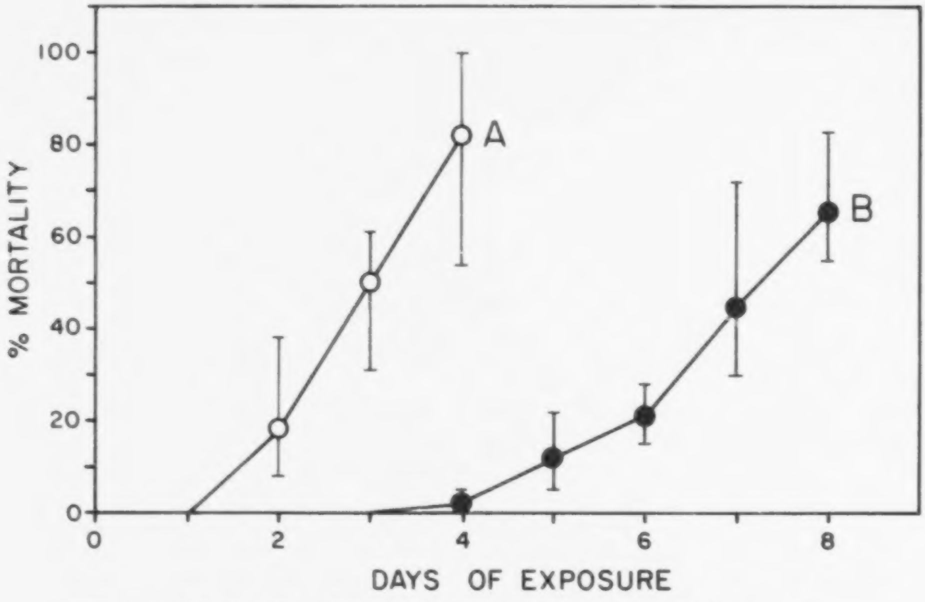

Figure 11-3. Batch variability in N. obsoletus larval survival upon exposure to "1.0" ppm fuel oil (WAF).

NOTE: Experiment "A" employed 15 larvae/vial and ran 4 days. Experiment "B" employed 20 larvae/vial and ran 8 days. The range of mortality observed in the 3 replicates run at each concentration is indicated.

Static exposures of larval *C. fornicata* ran only three days. No mortality was observed at any oil level tested. However, no larvae were observed swimming at "1.0" ppm by day two. Activity was normal at lower oil concentrations. Moreover, the guts of larvae at "1.0" ppm were empty of food by the second day, whereas veligers at lower oil concentrations continued to feed during the three-day experiment. In the flow-through exposure, veligers held at "1.0" ppm also stopped swimming by the second day of the experiment. These larvae were alive but emaciated by day four, and dead by day six. *Crepidula fornicata* larva had good survival at lower oil levels, although growth rates were affected as discussed below.

Gastropod Reproduction

Adults of *N. obsoletus* collected in February, 1976, and October, 1976, deposited their first egg capsules in laboratory control containers on 4/30/76 and 5/6/77, respectively, when the water temperature warmed to approximately 10°C. Sastry (27) also obtained egg capsules at 10°C from *N. obsoletus* collected in January at Beaufort, North Carolina.

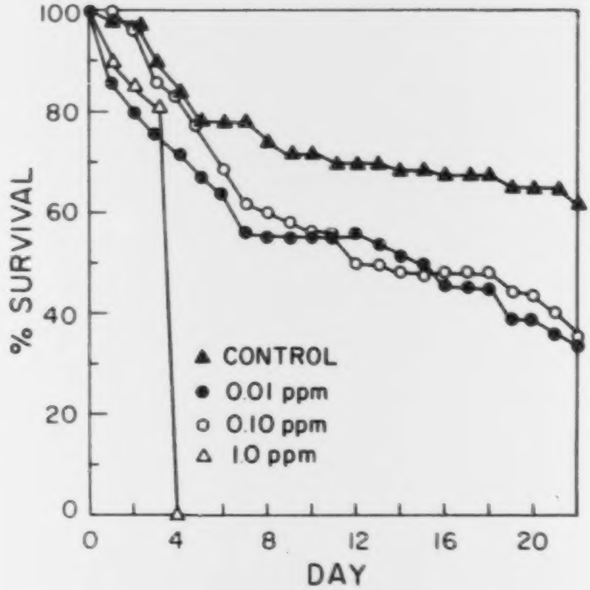

Figure 11-4. Survival of C. irroratus during development at different oil concentrations.

NOTE: Mean oil concentrations ± s.d. (N) were "0.01" ppm: 0.011 ppm ±0.009 (14); "0.1" ppm: 0.094 ppm ±0.027 (14); "1.0" ppm: 0.913 ppm ± 0.210 (14).

Onset of egg capsule deposition by snails exposed to "0.01" ppm and "0.1" ppm was delayed by about two weeks relative to controls in 1976, and delayed up to one week in 1977. Encapsulated embryos produced by these oil-exposed snails and transferred to control conditions developed to hatching without noticeable abnormality. In a mid-summer experiment, control individuals produced many capsules within three days of collection from the field, but those held at "1.0" ppm never deposited any capsules.

Egg capsule production is used here as an index of fecundity for both *N. obsoletus* and *U. cinerea*, since exposure to oil did not alter the average number of eggs per capsule (Table 11-2).

Egg capsule production by *N. obsoletus* held at "0.1" ppm may be reduced relative to control, and "0.01" ppm snails (Table 11-3, line "e"), although these data are insufficient for statistical analysis. Results are inconclusive due to the death of an unknown number of females during the test breeding period, precluding accurate calculation of individual fecundity.

Table 11-2. Effect of Exposure to No. 2 Fuel Oil (WAF) on the Distribution of Eggs Among Egg Capsules of N. obsoletus and U. cinerea.

		Nominal Oil Concentration	
Species	Control	0.01 ppm	0.01 ppm
N. obsoletus			
Mean oil concentration	0.0	0.021 ± 0.012(15)	0.086 ± 0.048(60)
No. capsules examined	8	8	8
Mean eggs/capsule ± s.d.	62.8 ± 14.8	61.5 ± 11.1	64.8 ± 11.6
F = 0.013 (P > 0.25)			
U. cinerea			
Mean oil concentration	0.0	0.016 ± 0.011(18)	0.067 ± 0.033(22)
No. capsules examined	20	20	20
Mean eggs/capsule ± s.d.	8.5 ± 1.5	8.6 ± 1.8	8.1 ± 2.5
F = 0.46 (P > 0.25)			

NOTE: F-values calculated by one-way analysis of variance. Oil concentration is given as mean ppm ± s.d. (N).

Table 11-3. Effect of Exposure to No. 2 Fuel Oil on Fecundity of N. Obsoletus.

	Control		Nominal Oil Concentration 0.01 ppm		0.10 ppm	
	A	B	A	B	A	B
a. Total capsules	11,892	6,771	6,442	10,983	7,509	7,419
b. No. live snails 6/3	33	34	34	28	33	37
c. No. live snails 8/17	31	28	33	28	30	35
d. No females 8/17	14	11	11	14	14	20
e. Maximum capsules/female*	849.4	615.5	585.6	784.5	536.4	370.9
f. Minimum capsules/female**	743.2	398.3	536.8	784.5	441.7	337.2

* Assuming all dead individuals were female (a/d)

** Assuming no dead individuals were female

NOTE: Each container initially held 35 snails. Two groups of snails were exposed at each oil level. Dates of exposure were 2/13/76 – 8/9/76. Measured oil concentrations are given in Figure 11-5.

Oil exposure modified the normal pattern of egg capsule deposition by adult *N. obsoletus* (Figures 11-5 and 11-6). Control snails tended to climb up the sides of the containers and deposit egg capsules mostly on the underside of the lid (Table 11-4). In the intertidal zone where spawning occurs, this behavior would contribute to placement of egg capsules into high-humidity environments where exposure of developing embryos to desiccation stress would be minimized (23). In contrast, *N. obsoletus* exposed to the "0.01" ppm and "0.1" ppm oil deposited capsules primarily on the container sides (Table 11-4). This effect was consistently most pronounced in May and June, at the beginning of the reproductive season (Figures 11-5 and 11-6). After 15-20 days, lid deposition began to increase for oil-exposed snails in both runs. However, lid deposition by control snails remained greater than that by

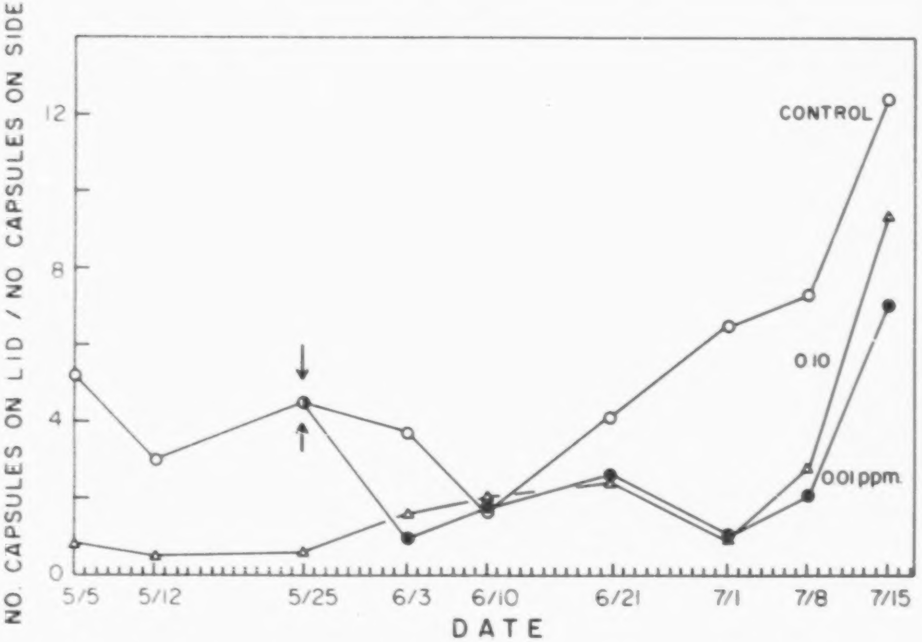

Figure 11-5. Influence of No. 2 fuel oil on egg capsule deposition behavior of N. obsoletus, 1976.

NOTE: Exposures were initiated 2/13/76. Each point represents data pooled from 2-4 replicate containers holding fifty snails each. The total number of capsules deposited were: 19,492 (control); 9,894 ("0.01" ppm); 11,287 ("0.1" ppm). Arrows indicate the transfer of two control containers to "0.01" ppm. Mean total petroleum hydrocarbon concentrations ± s.d. (N) at each nominal concentration were "0.01" ppm: 0.020 ± 0.008 (9); "0.1" ppm: 0.082 ± 0.044 (20).

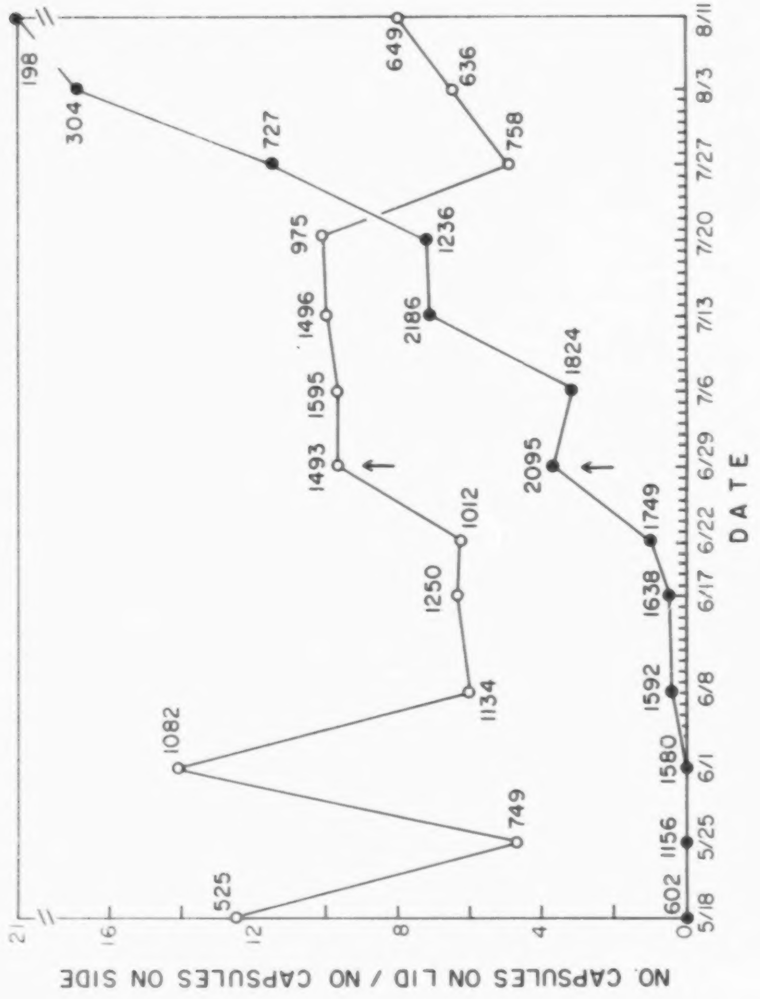

Figure 11-6. Influence of No. 2 fuel oil on egg capsule deposition behavior of N. obsoletus, 1977.

NOTE: Exposures initiated 10/22/76. Open circles represent data from control snails, closed circles represent data from snails held at "0.1" ppm. Mean oil hydrocarbon concentration at "0.1" ppm ± s.d. (N) was 0.077 ppm ±0.026 (34). Arrows indicate reversal of treatments.

Table 11-4. Placement of Egg Capsules by *N. obsoletus*.

Nominal Oil Level	Capsules on Lid	Capsules on Sides	Lid/Side Ratio	Oil Concentration
1976				
Control	19,092	3,570	5.3	0.0
0.01 ppm	7,215	2,456	2.9	0.0201 ± 0.008(9)
0.1 ppm	10,112	4,816	2.1	0.082 ± 0.044(20)
1977				
Control	6,393	852	7.5	0.0
0.1 ppm	3,445	6,967	0.5	0.081 ± 0.025(17)

NOTE: Summary of results obtained in 1976 (4/30 – 8/9) and 1977 (5/18 – 6/29). Oil concentrations are given as mean ppm ± s.d. (N).

oil-exposed snails, with one exception (i.e., 6/10/76). After transfer of two control containers to "0.1" ppm, lid deposition decreased and remained suppressed for some 30 days (Figure 11-5). In a similar experiment conducted the next spring, control containers were transferred to "0.1" ppm and containers held at "0.1" ppm were transferred to control conditions. Shifts in deposition patterns were again observed (Figure 11-6), although not until after four weeks for the former control group of snails.

With oyster drills (*U. cinerea*), there was no demonstrable effect of oil on fecundity when tested by one-way analysis of variance ($P > 0.25$), nor did exposure to "0.01" ppm and "0.1" ppm have any statistically significant effect on egg capsule placement. Egg capsule deposition behavior of oyster drills was affected by oil, however. No drills held at "0.1" ppm deposited capsules on the undersides of the container lids, in contrast to 13% to 14% lid deposition in 0.01 ppm and control treatments, respectively (Table 11-5).

Larval Growth

The influence of No. 2 fuel oil (WAF) on larval growth of three invertebrate species is summarized in Table 11-6. Growth of *N. obsoletus* larvae (μm shell length) was dramatically impaired at "0.01" ppm and "1.0" ppm, but was only slightly reduced at "0.1" ppm. This curious response pattern was observed in two experiments involving different hatches of larvae. In contrast, the effect of oil on larval growth of *C. fornicata* correlated positively with increasing oil concentration in the static experiments. Reduced growth was also evident at "0.1" ppm in the single flow-through experiment conducted. Growth of *C. irroratus* larvae, measured as change in mean dry weight/individual, declined relative to controls as development proceeded at the "0.1" ppm concentration. The dry weight of oil-exposed larvae was only 70.1 percent of control weight for the Stage IV zoea, and only 63 percent of control weight for the Stage V zoea. However, these same *C. irroratus* larvae exhibited no differences in molting frequency or in carapace length with respect to control individuals. *Cancer irroratus* larvae in all sublethal oil treatments (i.e., all below "1.0" ppm) reached the megalops stage in 25-28 days.

DISCUSSION

The concentration of hydrocarbons lethal to larvae is generally believed to be about one-tenth of the concentration lethal to adults (14, 21). In our study, a nominal concentration of 1.0 ppm was lethal to adults and larvae alike, although 50% mortality of larval *N. obsoletus* occurred much sooner for larvae than for adults. Exposure to "0.01" ppm and "0.1" ppm produced mainly sublethal effects in both larvae and adults of this species.

Table 11-5. Effect of Exposure to No. 2 Fuel Oil (WAF) on the Egg Capsules Deposition Patterns of *U. cinerea*.

		Nominal Oil Concentration		
Substrate	Control	0.01 ppm	0.1 ppm	F
Underside of lid	12.9%	14.0%	0.0%	2.0
Bottom Substrates (rock and shells)	58.8%	53.2%	40.0%	0.3
Sides	28.3%	32.8%	60.0%	1.1
Total No. Capsules Deposited	583	808	477	

NOTE: Table entries are percent of total egg capsules deposited on each surface. Data are means of three replicates at each concentration. F-values (d.f. = 2, 6) calculated by one-way analysis of variance on percentages of total egg capsules deposited. Oil concentrations are given in Table 11-2.

Table 11-6. Effect of Exposure to No. 2 Fuel Oil (WAF) on Larval Growth Rates, Calculated as (Control Growth - Experimental Growth)/Control Growth x 100%.

		Percent Reduction in Growth Nominal Oil Concentration			
Species	Days Exposed	0.01 ppm	0.1 ppm	1.0 ppm	No. Larvae/ Treatment
N. obsoletus	4	44.7%	7.9%	94.7%	45
N. obsoletus	8	52.4%	6.1%	97.6%	60
C. fornicata	3	11.7%	69.1%	100%	60
C. fornicata	3	–	70.6%	100%	40
C. fornicata (flow-through)	14	–	44.8%	–	50
C. irroratus	22	–	37.0%	–	75

NOTE: Growth of veliger larvae was measured in terms of shell length (μm) while growth of crab zoea was measured in terms of dry weight (μg). All experiments were conducted under static conditions, except as indicated. Measured hydrocarbon concentrations are given in Table 11-1 and Figure 11-4 legends. Each entry is based on the pooled data from 2-3 replicates at each concentration.

Experiments on pollutant toxicity are usually conducted under constant conditions of light, temperature, and salinity. The results of such experiments may not be applicable to the field, where environmental conditions vary. A case in point is the observed seasonal variation in oil toxicity to adult *N. obsoletus*, with toxicity being accentuated in winter. Egg capsule deposition patterns of oil-exposed *N. obsoletus* also showed temporal variability. Patterns were least like those for control snails when water temperature was low at the beginning of the breeding season, but changed substantially as water temperatures rose. Similarly, Krebs and Burns (17) have observed that fiddler crabs (*Uca pugnax*) exposed to No. 2 fuel oil in the field show abnormal behavior only at temperatures near the lower limit of their normal range of activity.

There are several possible explanations of the greater toxicity of this oil at low temperatures. Low temperature may directly increase the relative concentration of the more toxic oil fractions present in the water, either through altered solubility, volatization, or shifts in bacterial activity. This hypothesis is currently being explored. It is also possible that seasonal changes in toxicity result from changes in the physiological state of the animals and/or from additive effects of low temperature and oil stress.

There is currently little information on how petroleum hydrocarbons enter aquatic animals, but recent evidence indicates that uptake of oil through ingestion of contaminated food may be at least as important as diffusional uptake (8, 11, 18). This is consistent with our observations of higher mortality of adult *N. obsoletus* in the presence of sediment. This occurred only during the summer, when *N. obsoletus* is actively deposit-feeding (30). The sediment effect did not occur during the winter, when the snails are inactive.

We observed several sublethal effects on invertebrate reproduction, including possible reduction in the fecundity of *N. obsoletus*. Although there was no alteration in the number of eggs per capsule, the number of capsules produced appeared to decline. One possible variable influencing egg capsule production may be date of initiation of oil exposure relative to the onset of oogenesis. In this study, *U. cinerea* were exposed to hydrocarbons after gametogenesis was completed, and egg capsule depostion was already underway, which might explain the absence of a fecundity response for this species. More data are needed to resolve this issue.

The observed alteration of egg capsule deposition behavior with respect to substrate orientation has significant ecological consequences for *N. obsoletus*. The egg capsules and embryos of *N. obsoletus* are not well adapted for deposition in the exposed intertidal zone; successful pre-hatching development of this species is apparently dependent instead upon the proper placement of the capsules on substrates (23). Interference with normal patterns of egg capsule deposition behavior could substantially increase pre-hatching mortality from desiccation stress (23).

The reduction in larval growth rate observed at sublethal oil concentrations could result from increased energy expenditure, decreased ingestion rate, decreased assimilation efficiency, or a combination of these factors. Present evidence suggests that the reduced growth observed was due at least in part to reduced food intake. Larvae of *C. fornicata* and *N. obsoletus* held at "1.0" ppm ceased feeding at least one to two days before they died. The larval guts of *C. fornicata* were empty of food by the second day of each experiment, even though the velar lobes remained extended and ciliary activity was observed. Tissues in these individuals became dramatically shrunken within several days after initiation of exposure to oil. Veligers held at oil concentrations of "0.10" ppm showed no such morphological abnormality, but preliminary experiments (Pechenik, unpublished) reveal decreased ingestion rates at this concentration, relative to ingestion rates of control larvae.

It is not yet possible to precisely predict the threshold oil concentrations at which lethal or sublethal effects occur. The potential for seasonal changes in oil toxicity has already been discussed. Moreover, most laboratory experiments conducted to date, including many in the present study, have used static exposures in which the dosing medium is replenished at one to two day intervals. Due to loss of volatile fractions from these aqueous mixtures, the initial hydrocarbon concentrations cited represent only maximum concentrations which the animals experienced during a test (6, 32). Atkinson *et al* (4) reported that 90 percent of the benzene initially present in a test solution is lost from undisturbed cotton-plugged flasks within a 24 hour period. Our containers were kept tightly sealed in the static experiments, minimizing such loss. Some loss of hydrocarbons through volatilization could have occurred during transfer of the medium from the flow-through tanks to the experimental containers, however. Finally, oil concentrations are generally reported as total hydrocarbon content, as measured by infrared spectrophotometry. Yet, toxicity to animals is probably due to only a small fraction of the hydrocarbon compounds present in the water accommodated fraction used (17), a fraction which can vary qualitatively and quantitatively over the period of an investigation. The concentration of specific oil fractions present during an experiment is generally unknown. Better control and analysis of oil exposures conditions are needed if we wish to accurately determine threshold concentrations of oil which are toxic to marine organisms.

ACKNOWLEDGEMENTS

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EXTRACTION OF ENVIRONMENTAL INFORMATION STORED IN MOLLUSCAN SHELLS: APPLICATION TO ECOLOGICAL PROBLEMS

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ABSTRACT

Ecological stress, when broadly defined, is responsible for most, if not all, growth patterns within the molluscan shell. As the type of pattern deposited is largely a function of the specific biological or environmental stress involved, considerable ecological information is stored within the exoskeleton. The resulting record is in the form of either (1) microstructural growth increment sequences or (2) changes in the shell structural type (e.g., nacreous, prismatic, crossed-lamellar, etc.) or relative proportions of structures within the shell.

Microstructural growth increments, heretofore interpreted as resulting from varible despositional rates of calcium carbonate and organic matrix, are viewed as refections of periodic shell dissolution-deposition cycles.

Changes in the type of crystalline structure deposited under various environmental conditions within the inner shell layer of several species of bivalves have been defined. During periods of extreme ecological stress, such as prolonged exposure to sub-freezing temperatures, extensive dissolution and "reworking" of this inner layer occurs in a number of species.

Extraction of environmental information recorded within the shell is facilitated through examination of polished thin sections, acetate peels, fractured shells, polished and etched shell sections, and growth surfaces using polarizing, optical, and scanning (and, occasionally, transmission) electron microscopy. Application of these techniques to long-term monitoring of ecologically stressed environments is discussed.

INTRODUCTION

Ecology has been defined as the study of relationships between organisms and their environment (28). In functioning ecosystems it is possible to make direct observations of these relationships in real time. Organism-environment relationships may not always be directly measured, however, requiring an indirect or deductive approach; for instance, we may wish to assess the effect of a storm, pollution event, change in salinity, temperature, etc. on a species population after the event has taken place. In the absence of data about pre-disturbance rates of growth, death, and reproduction, we are totally dependent on indirect techniques. This kind of after-the-fact problem is common in paleoecology and promises to be an increasingly important approach in pollution biology[1].

Subject matter of the present article has been extracted and condensed from initial drafts of a manual which is currently being prepared for the Environmental Protection Agency[2]. The purpose of this manual is to bring together and organize paleoecological literature so that it may be of use to the pollution biologist confronted with after-the-fact monitoring[3] problems.

The Skeletal Record

Skeletonized organisms provide an opportunity for deducing ecologic relationships in the past. The skeleton often contains a record of dynamic life and death processes, and provides both *ontogenetic* and *demographic*[4] information. *Ontogenetic* data are related to the life history of an individual. Growth rates may be resolved to a high level of resolution from mineralized tissue showing growth banding correlated with lunar and/or solar cycles, or seasonal changes in water temperature, salinity, day length, primary productivity, etc. Biological events, such as season of reproduction and death, may also be recorded. *Demographic* data are related to population structure and its maintenance; growth, mortality, recruitment, and migration. The unit of study is a single species population. In the present article, we will limit our discussion to extraction of *ontogenetic* data.

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[1] Paleoecologic research over the past decade has developed many techniques for reconstructing paleoenviroments (30-32). Much of this literature is unknown to neontologists.

[2] Preparation of the manual is supported by Environmental Protection Agency grant R804-909-010.

[3] The term monitoring is used here to describe reconstruction of an organism's history of growth, reproduction, and mortality as preserved in its skeletal parts. Inferences about environmental causes for the observed record are, by definition, indirect and deductive.

[4] Demography, taken literally, means writing about the people (Gr. demos, the people + to write). The term was originally used to describe statistical studies of human populations; births, deaths, marriages, etc. We use demography in a broader sense; the statistical description of populations of any taxonomic group.

Although all organisms with either an exoskeleton or endoskeleton can potentially provide ontogenetic data, bivalve molluscs are the most universally used group for obtaining these types of information for three reasons:

(1) Most members of the Class Bivalvia are preservable, and are common faunal elements in both recent and fossil assemblages. Many species are present in areas impacted by pollution and are represented in both early and late stages of ecological successions following seafloor disturbance.

(2) Preparation of the shell for obtaining ontogenetic information is easily done. This involves sectioning or fracturing the shell along a plane passing from the oldest part of the shell, the umbo, to the growing edge along the maximum axis of growth (30, 32). Coiled or otherwise torqued shells (e.g. gastropods) make this technique impossible with present methods.

(3) Most research relating shell parameters to environmental conditions is based on bivalves.

Data from Living and Dead Molluscs

The relationship of a species to its environment has been conceptualized in the niche model (28). The species of interest is able to grow and reproduce as long as the organisms' functional range (biospace) is not exceeded by the ambient environment. Not all parts of the realized biospace promote equal growth or fecundity. Different combinations of niche parameters will be manifested in changed rates of growth, survivorship, or reproductive success. All of these manifestations are capable of being preserved within the shell. We therefore have a record of an organism's responses to changing niche conditions preserved in shells of individuals, composing either the living or death assemblage.

Suboptimal niche conditions can be thought of as *ecological stress*. Ecological stress is responsible for most, if not all, growth patterns within individual shells. In this regard, an ecological stress, such as a pollution event, can be assigned dimensions in both space and time. These dimensions are important when considering species appropriate for establishing after-the-fact relationships. Ideally, the spatial distribution of a species should overlap and extend beyond the affected area. Populations falling outside of the polluted area can be used as control or reference populations. If the pollution event is lethal to part or all members of the population occupying the affected seafloor, after-the-fact study will include a comparison of both living and death assemblages. If the effect is sublethal, living assemblages alone will be used.

The duration of the pollution event should be considered relative to the mean life span of individuals (turnover rate). Again, the ideal situation is one where the species overlapping the affected area is one with a low turnover rate, and a life span that is long relative to the duration of the pollution event. If the ecological stress is sublethal, a record of growth before, during, and after the stress event, may be recorded within the living population, and can be compared with that of the reference population outside the affected area. If the stress results in high mortality, the death assemblage may be all that remains to document the event.

MOLLUSCAN GROWTH PATTERNS

Environmental information is stored within the molluscan shell in the form of either (1) microstructural growth increment sequences or (2) changes in the shell structural type (e.g. nacreous, prismatic, crossed-lamellar, etc.) or relative proportions of structures within the shell. These two distinct types of records and their usefulness in ecological studies are discussed below. Much of this discussion is taken directly from a recent article by Lutz and Rhoads (26).

Microstructural Growth Patterns

During the past decade, numerous workers (2-4, 13, 14, 30-32) have described microstructural increments within the molluscan shell. As a result of marked periodicity associated with many of these structures, they have proved useful in geophysical studies for defining changes in the earth's rotational rate (3, 29-31), in ecological and paleoecological studies for assessing the effects of various biological and environmental stresses (9, 14, 18, 30, 32), and in archaeological studies for reconstructing migration patterns of prehistoric hunter-gatherers (6, 7, 19). When shells are viewed in cross-section (procedural details outlined in Methods section below), these microstructural patterns are seen as alternating bands of shell material ranging in thickness from 10^0 to 10^2 μ.

Many, if not all, microstructural periodicity structures within the molluscan shell are a reflection of variations in the relative proportions of organic material (conchiolin) and calcium carbonate (aragonite or calcite). Alternation of calcium carbonate-rich layers and organic-rich regions or lines has been well documented for numerous recent and fossil species through detailed studies of shell thin sections, acetate peels, and polished and etched surfaces, employing polarizing, optical, and scanning electron microscopy (see Methods section). "Daily" growth increments have been reported by several workers (2, 13, 14, 18, 30-32). These "daily" lineations were originally interpreted as reflections of solar time (13, 14, 17, 30, 31). Recent studies, however, have revealed a complex relationship between incremental growth, and lunar and solar cycles.

Although a one-to-one correspondence has not been established, the deposition of increments in bivalves is highly correlated with shell valve movements (27, 30, 35, 36). As the valves of many species are generally closed during low tide, and open during high tide, a high positive correlation also exists between the number of increments and the number of tides to which an organism has been subjected. While valve-movement rhythmicity is generally most pronounced in intertidal individuals, subtidal specimens of at least one species (*Mercenaria mercenaria*) exhibit biological rhythms in relative harmony with the tidal cycle. There is general agreement among growth line workers that when the valves are open and the organism is actively pumping, a layer is deposited which is rich in calcium carbonate relative to adjacent shell material. The origin of alternating layers or lines relatively rich in organic content has recently been theorized by Lutz and Rhoads (26). The following few paragraphs summarize this theory which is based on recent studies of molluscan anaerobiosis and mechanisms of shell formation.

During aerobic metabolism, molluscs deposit calcium carbonate, in the form of either aragonite or calcite, together with organic material, resulting in shell construction. Such metabolism is usually highly correlated with periods of active pumping during high tide in well-oxygenated waters. As the concentration of dissolved oxygen falls, such as in the microenvironment created by the organism during periods of shell closure, anaerobic respiratory pathways are employed and levels of succinic acid (or other acidic end-products) within the extrapallial fluid rise. The acid produced is gradually neutralized by shell calcium carbonate, leading to increased levels of Ca^{++} and succinate (or other end-products) within the extrapallial and mantle fluids (8). As a result of this decalcification, the ratio of relatively acid-insoluble organic material to calcium carbonate increases at the mantle-shell interface. One need not invoke the complication of increased concentration of organic material in a given volume, although a collapse of unsupported matrix structures or movement of the mantle as a compensatory response to the increased mantle-shell distance could result in increased concentrations of freed organic material in specific regions of the extrapallial fluid. With the return of oxygenated conditions and resumption of aerobic metabolism, and assuming shell deposition during this post-anaerobic period proceeds via a process similar to that occurring immediately prior to anaerobiosis, deposition of calcium carbonate and organic material within an area already containing organic material should result in an increase in the organic/ $CaCO_3$ ratio within the specific shell region. The end-product of this process, from a strictly structural viewpoint, is one growth increment.

Methods

Microstructural increments can be studied in thin sections of shell material, in acetate peel replicas of acid-etched shell sections, or under the scanning electron microscope (fractured or polished and etched shell sections).

Acetate peels are the easiest and most rapid method of preparation for examination of most molluscan shells. The basic method of preparation, as outlined by Rhoads and Pannella (32), is as follows:

Shells are embedded in a block of epoxy resin (e.g., Epon 815 resin with DTA hardener, 10:1 ratio, under vacuum; Miller-Stephenson Chemical Company, Danbury, Connecticut) to avoid shell fracture during sectioning. The plane of the cross-section passes from the umbo to the shell edge along the axis of maximum growth (30, 32). This cut is oriented so that growth increments intersect the plane of the section at right angles. The cut shell surface is polished sequentially with 350, 600, and, finally, 2600 or 3000 grade carborundum grits. The polished surface is then etched with 0.1 N HC1 for periods varying from a few seconds to a few minutes. Optimal etching time is related to shell structure, mineralogy, organic content, and state of preservation. It is recommended that a series of test etching times be carried out to determine optimum etching periods for a particular set of specimens.

Etched shell surfaces are flooded with acetone, and a piece of sheet acetate is applied to the etched shell surface and weighted to avoid bubble formation. After the acetone (solvent) has evaporated (approximately 30 minutes), the acetate is removed from the shell and examined under the microscope (or used as a negative by placing directly in a photographic enlarger and printing). This technique yields excellent results for most species.

Thin-sections are necessary for the examination of growth increments which are not structurally discontinuous, but instead recognizable only by dark and light color bands (32). For example, the growth increment boundaries in the deep-water species, *Nucula cancellata* and *Calyptogena ponderosa*, are indistinct and recognizable only by color variations of the bands, each band consisting of one dark and one light layer. The initial procedure for making thin-sections is the same as that for preparation of acetate peels, however, after the cut shell surface has been polished, it is glued to a glass slide using epoxy resin. The majority of the embedded shell and remaining embedding material is cut away using a diamond rock cutting saw, and the new exposed surface is polished sequentially until a 0.03 mm thick section of material is left on the slide. A cover slip is glued onto the newly polished surface using epoxy resin, and the material examined using optical or polarizing microscopy. Thin sectioning of shells is difficult, because shells tend to fracture when sectioned and the micro-growth lines are obscured. In addition, it is difficult to avoid the formation of bubbles beneath the coverslip which obscure features. Equipment for preparing thin sections is available in rock preparation laboratories found in most geology departments. Alternatively, there are commercial firms (e.g., Rudolf von Huene, Pasadena, California) that will prepare thin sectioned material.

Ecological Applications

Several workers (9, 14, 18, 30-32) have suggested that information about physiological and environmental conditions may be recorded and stored in molluscan shells. Various studies in which workers have used microstructural increments within shells to extract such information are discussed below under appropriate sub-headings.

Seasonal Cycles

Seasonally caused annual growth rates and patterns are observable in all bivalves collected in climatic zones, ranging from cold-temperate to sub-tropical. In many species, as winter approaches, there is a gradual slowing down of the deposition rate, and the microstructural increments become gradually thinner. This slowing down of growth in the autumn culminates in a marked depositional break at the time of the first freeze. These depositional breaks are characterized by indentations of the outer shell layer, a dark band of organic-rich shell material extending downward from the base of the indentation, small daily growth increments on either side of the break, and a change in the shell structure near the break (9). These winter breaks may not be as marked in specimens living subtidally (32), although *Mercenaria mercenaria* from water depths of eight meters clearly show a distinct winter break (9).

Through careful examination of microstructural patterns, Farrow (13) found that part of a population of the shallow subtidal cockle, *Cerastoderma edule*, from the Thames estuary in England, stopped growing during winter due to sub-zero temperatures. Tevesz (34) observed that *Gemma gemma* grew very little in the winter. Growth increments were very closely spaced and the inner shell layer had a brownish hue. During the summer, *G. gemma* grew rapidly; microstructural increments were widely spaced, and the inner shell layer was clear and translucent in appearance.

Through an examination of numerous acetate peels, Evans and LeMessurier (12) were able to demonstrate striking winter growth rate differences between two sympatric species of bivalves. They found winter growth of the rock-boring clam, *Penitella penita*, to be approximately 75 percent of the summer growth rate, while growth of the cockle, *Clinocardium nuttalli*, which inhabited a neighboring mud flat, was slowed by as much as a factor of 19.

In addition to rhythms based on periodic environmental fluctuations, biological rhythms, such as breeding periods, are also reflected in growth increment clustering. "Breaks" due to spawning events are less severe than winter breaks. They are preceded by little or no slowed growth, and recovery is more rapid than after winter breaks. In most bivalves, spawning occurs during the summer, sometimes more than once a year. In *Mercenaria mercenaria*, reproductive breaks do not occur until the second year of growth (32).

Semiperiodic and Random Events

Depositional breaks in bivalves also result from semiperiodic or random events such as storms, unseasonable temperatures, attacks by predators, and environmental pollution disturbances. The irregular nature of such events makes them easily separable from periodic or cyclical shell-secretion rhythms (18, 30, 32).

Storm breaks, a common feature, may have different characteristics depending on the severity of the storm and depth at which the bivalves live below the water surface. In any case, these breaks appear suddenly and are followed by a rapid return to increments of pre-storm width (9). Shuster (33) noted that during storms, silt became trapped between the mantle and the shell in *Mya arenaria* and was subsequently incorporated into the shell. Trapped silt within shell indentations formed by storm breaks has also been observed in *Mercenaria mercenaria* (9, 18).

The Season, Age, and Frequency of Reproduction and Death

Growth patterns can supply detailed information on the age of individuals at time of death and their season of death. The age at death will be:

$$\mathrm{Ad} = \frac{N_s + N_w}{2}$$

where Ad is age at death and N_s and N_w are, respectively, the number of summer and winter bands in the shell (32). The season of death is determined by relating the position of the last increment at the margin of the shell to the seasonal growth pattern. For example, a margin preceded by a complete summer depositional record represents death in early fall. A margin which follows a long period of winter growth represents late winter or early spring death. Often, the shell margin is preceded by a few days of growth slowdown, and comparison of several individuals may be necessary in order to determine if the slowing down in growth represents a moribund condition prior to death, or is related to seasonal changes (32). By counting the number of increments in a dead shell, it is also possible to relate the season of death to absolute age at death.

The age at sexual maturity and season of reproduction can be determined by relating the position of spawning breaks to absolute age and seasonal pattern of growth. An illustration of the usefulness of growth patterns in determining age and season of reproduction is given by Rhoads and Pannella (32). They examined a population of *Gemma gemma* from an intertidal muddy sand flat on Long Island Sound. Summer growth patterns in *G. gemma* consisted of thick increments (7-25 μ) and were readily distinguished from winter ones which were thin (1-3 μ). A period of decreased growth was seen in shell sections and was interpreted by them as reflecting reproductive events which occurred at the beginning of summer deposition. These thin increments, if related to spawning, should be associated with a spawning break in the shell margin. Rhoads and Pannella (32) determined that the periods of highest stress and mortality were different for juvenile and mature bivalves. Specimens 3.2 mm (generally less than 6 months old) died with greatest frequency from summer to mid-autumn. Older individuals died primarily in late fall or early winter.

Ontogenetic Records of Environmental Change.

In addition to episodic and periodic events, variations in environmental parameters including food supply, the type of substratum, salinity, oxygen content, turbidity, agitation, temperature, and population density can influence growth of bivalves. Hallam (15) reviews these various environmental parameters as causes of stunting and dwarfing in living and fossil marine benthic invertebrates. Several studies conducted within the past few years have used microscopic growth increments within shells to define the effects of various environmental perturbations on bivalve growth. Rhoads and Pannella (32), for example, through careful examination of both acetate peels and thin sections, have demonstrated that examination of both acetate peels and thin sections, have demonstrated that *Mercenaria mercenaria* grows faster in sandy sediments than in mud when other variables are eliminated. Farrow (13) used microstructural growth increments within the shell of *Cerastoderma edule* to illustrate that dense populations of the cockles had a much shorter growing season than sparse populations. An inverse relationship between individual size and population density of cockles was also noted. In a subsequent study, Farrow (14) used growth increments within the outer shell layer of this species to demonstrate that individuals living high in the intertidal zone were stunted. The higher shore cockles were situated near the high water mark, and, consequently, were aerially exposed for several days during neap tides. Following neap tide deceleration, there was a resumption of vigorous growth. Many of the high intertidal cockles were some two-thirds the size of individuals lower in the intertidal zone, where growth was more continuous.

In a recent study, Kennish and Olsson (18) studied the effects of thermal discharges on the microstructural growth of *Mercenaria mercenaria* in Barnegat Bay, New Jersey. They found that clams from within a mile and a half of the mouth of Oyster Creek, which carries the heated effluent from the Oyster Creek Nuclear Power Plant, had a much higher number of breaks in their shells, thinner shells, and slower summer growth than did clams farther from the river. Counting the growth increments back from the shell margin, they determined that many of the breaks occurred concurrently with rapidly decreasing water temperatures, resulting from abrupt shut-downs of the power plant, or rapidly increasing temperatures associated with abrupt renewal of plant operations. The growth rate of *M. mercenaria* generally increases with increasing temperatures and peaks between 20-24°C; Haskin (communicated to Kennish and Olsson) found decreased growth above 26°C. The thermal effluent raised the water temperature in areas around the mouth of Oyster Creek 3-5°C above ambient. Kennish and Olsson (18) also suggested that the thermal effluent may be adversely affecting physiological functions other than growth. At the station nearest the effluent, no spawning breaks were observed within the shells, while they were seen in specimens from all the control sites.

Shell Structural Changes

In addition to changes in patterns of microstructural growth increment sequences, changes in the type of crystalline structure deposited under various environmental conditions have been observed within the shells (particularly within the inner shell layers) of numerous species of bivalves. Dodd (10) described environmentally-controlled variation in the relative proportions of nacreous and calcitic prismatic structures within the innermost shell layers of *Mytilus californianus*. Lutz (23) found annual variation in the thickness of nacreous laminae within the inner shell layer of *Mytilus edulis*, and suggested that such variation might be growth rate and/or temperature dependent, with relatively fine laminae being formed with increased growth rate and rising temperatures in the late spring. Bryan (5) examined the effects of oil spill remover (detergents) on the shell of the intertidal gastropod, *Nucella lapillus*, following the Torrey Canyon spill in March of 1967. The addition of toxic detergent BP 1002 applied to the Kuwait crude oil spill was effective in temporarily sealing the shell edge by continuing the inner nacreous layer to the outer surface. Subsequent shell growth on thin nacre produced a growth mark and lines of weakness in the shell. Kennish and Olsson (18) observed transgressing regions of crossed-lamellar structure within the outer shell layer of *Mercenaria mercenaria* associated with shell deposition occurring during periods of extreme ecological stress (winter freezes, high summer temperatures, and thermal shocks from abrupt changes in operations of a nuclear power plant). Farrow (14) noted similar transgressing regions of crossed-lamellar structure within the outer layer of *Cerastoderma edule* associated with winter growth, particularly in specimens sampled from high elevations (highly stressed environments) within the intertidal zone.

Lutz and Rhoads (26) have recently presented evidence that structural changes within the shells of certain bivalve species may reflect periodic dissolution and "reworking" of primary depositional structures during periods of extreme environment stress. Here, alternating periods of aerobic and anaerobic metabolism provide the driving forces for shell deposition and dissolution, respectively. Parallel annually-formed sub-layers of nacre and simple aragonitic prisms (24, 25) within the inner shell layer of the Atlantic ribbed mussel, *Geukensia demissa* (Figure 12-1), for example, were interpreted as reflective of seasonal metabolic changes. In populations from Gulf of Maine waters, nacre deposition was restricted to the relatively warm months of the year (24, 25). During both the fall and spring, nacreous tablets on the inner shell layer growth surface became smaller and less regular, showing visible signs of erosion in the form of marked pitting and "hollow crystals", as well as increased proportions of fine-grained structures. Differential dissolution of calcium carbonate and organic material was also often observed at the inner layer growth surface during these seasons (Figure 12-2). During the colder months of the year (January – March, with water temperatures below $3^{o}C$), shell erosion became visible to the naked eye, the entire inner shell surface often presenting a chalky white appearance. Ultrastructurally, this surface appeared uniformly fine-grained or "homogeneous" (Figure 12-3). Similar visible erosion has been reported in *Mercenaria mercenaria* after long periods of valve closure (11). The ability of *G. demissa* to endure anaerobiosis for extended periods has been well documented (20, 22), as has the relative increased efficiency in this species of some of the citric acid cycle enzymes in an anaerobic direction (16). The observed shell erosion may well be a reflection of buffering of acid end-products from anaerobic metabolism during the colder months, when oxygen transport into the cells should theoretically be reduced relative to that occurring at higher temperatures (21). Wibur (37) has suggested that during periods of "adverse environmental conditions", shell decalcification may predominate over growth. The often-seen gradation in fractured, as well as polished and etched, vertical shell sections of *G. demissa nacreous laminae* into finely grained structures (suggestive of massive erosion), instead of regular prisms, (Figure 12-4) tends to support this view.

SUMMARY

(1) Environmental and biological events are recorded in the molluscan shell in the form of small-scale growth increments and/or changes in shell structure.

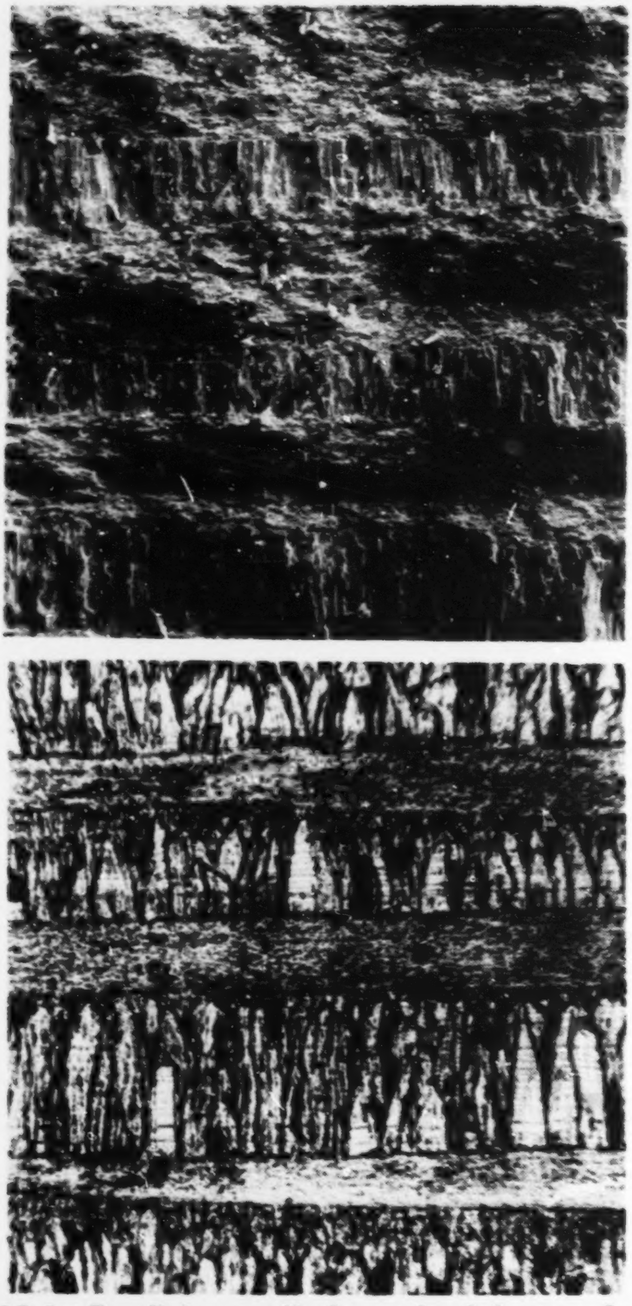

Figure 12-1. Parallel annually-formed sub-layers of nacre and simple aragonitic prisms within the inner shell layer of the Atlantic ribbed mussel, Geukensia demissa.

NOTE: (A) Scanning electron micrograph of vertical fracture surface x240.
(B) Acetate peel of polished and etched longitudinal shell section x400.

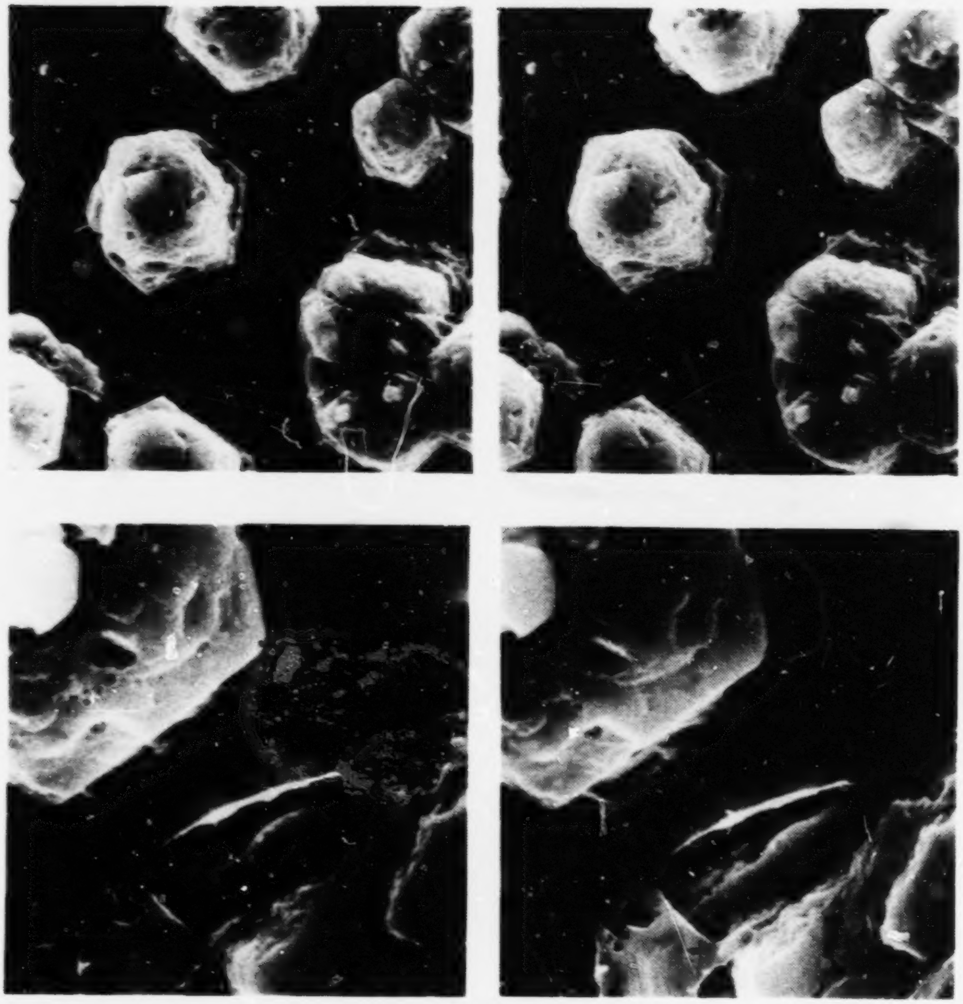

Figure 12-2. Scanning electron micrographs of the inner shell layer growth surface of Geukensia demissa showing natural shell dissolution.

NOTE: The differential solubility of calcium carbonate and organic matrices is apparent. Stereo pairs were taken with a 6° angular displacement between exposures. (A) x5000. (B) x20000.

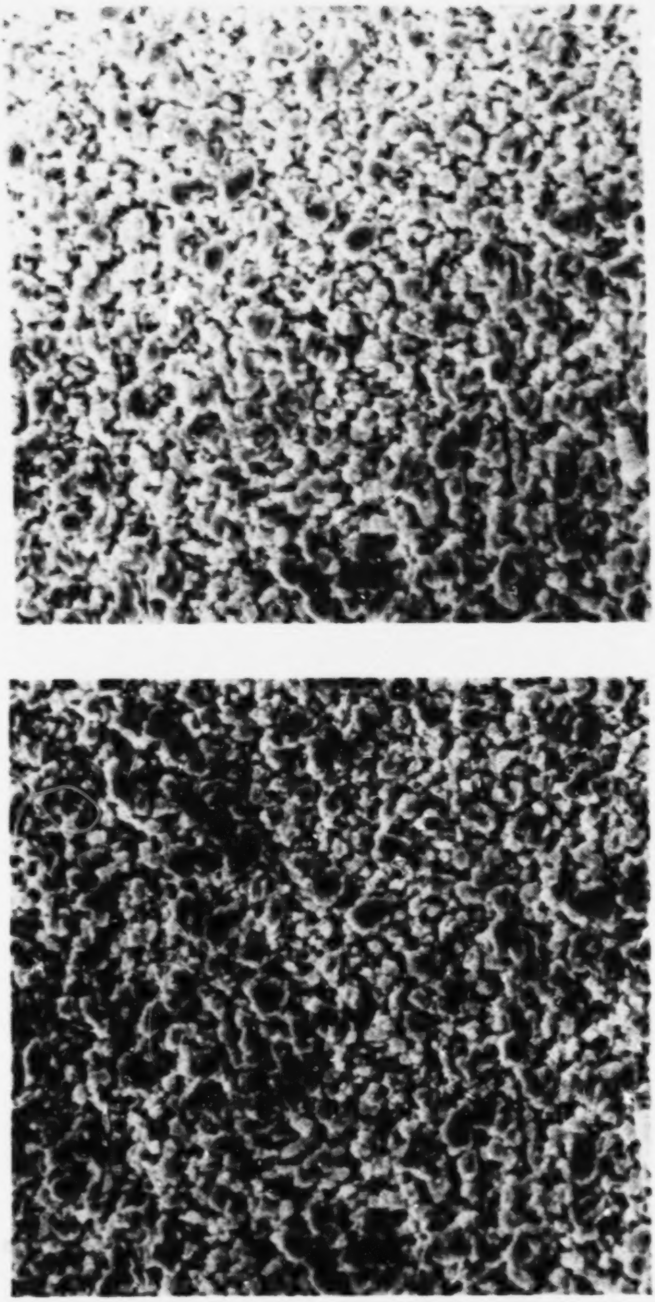

Figure 12-3. Scanning electron micrographs of the inner shell layer of Geukensia demissa showing fine-grained structures reflective of extensive shell dissolution during the colder months of the year (February sample).

NOTE: Stereo pair was taken with a 6° angular displacement between exposures. x3000.

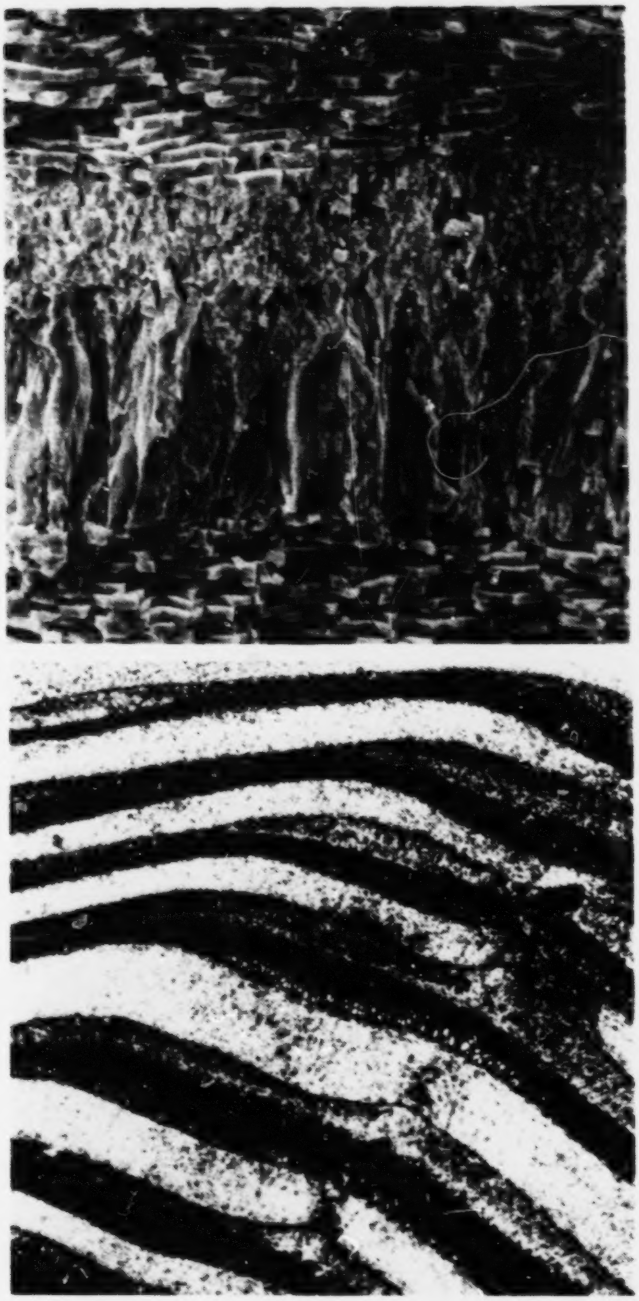

Figure 12-4. The inner shell layer of *Geukensia demissa* from Gulf of Maine waters.

NOTE: (A) Scanning electron micrograph of vertical fracture surface showing gradation of nacreous tablets into fine-grained structures at top and gradation of prisms into nacre at bottom x2000. The most recently deposited crystals are at the bottom of the micrograph. (B) Acetate peel showing similar gradations of nacre into fine-grained structures and prisms into nacre x125. Again, the most recently deposited crystals are at the bottom of the micrograph.

(2) Alternating aerobic-anaerobic metabolic cycles are proposed as the physiologic mechanism for forming shell periodicity structures. Aerobic respiration is associated with shell calcification. Shell closure, accompanied by anaerobic metabolism, results in shell decalcification; acidic end-products are neutralized by dissolution of shell calcium carbonate.

(3) Shell growth patterns can be easily studied by preparing shell thin sections or acetate peel replicas of acid-etched shell sections. Scanning electron microscopy of fracture or polished and etched shell sections can also be employed.

(4) Patterns of growth increment sequences and shell structural changes are related to seasonal climatic cycles and, on shorter time scales, to lunar and solar periodicities. Semiperiodic or random events, such as storms, sedimentation events, or biological events (e.g., reproduction) are superimposed as "noise" on the geophysical cycles. Causal effects for this "noise" can be deduced by detailed studies of the growth record.

(5) Shell growth patterns have proven useful in paleoecologic reconstructions. Detailed analysis of these patterns also promises to be an efficient manner in which to conduct after-the-fact or retrospective monitoring studies of pollution events.

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LABORATORY CULTURE OF MARINE FISH LARVAE AND THEIR ROLE IN MARINE ENVIRONMENTAL RESEARCH

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ABSTRACT

The capability to predictably culture marine fish larvae beyond embryonic and yolk-sac stages has been developed during the past 15 years. This has led to advances in our understanding of how environmental variables affect survival and eventual recruitment of fishes. Most marine fish larvae are planktonic carnivores and consume living prey less than 150 μm in breadth when they first feed. The most important advance in culture technology was the determination of kinds and concentrations of prey that enable larvae to survive and grow at predictable rates, permitting ecological, physiological, and behavioral research to be undertaken. Prey concentrations necessary for growth and survival of some typical marine teleost larvae, usually range from 10^1 to 10^3 per liter. Best survival rates, fastest growth, and lowest variability, have been obtained at the 10^3 per liter concentration. Growth efficiencies and food consumption by marine fish larvae are comparable to other predatory zooplankton. Some knowledge about effects of predation on marine fish larvae survival has been obtained, but further study is necessary, especially to determine how environmental factors modify predator effects. Some areas of environmental research, using cultured marine fish larvae, are reviewed. These include the roles of physical and chemical variables, other than man-induced environmental perturbations, and some effects of environmental change caused by man's encroachment upon and alteration of marine habitats. Other important advances include development of field bioassay methods to determine if plankton standing stock can support fish larvae; development of biochemical and histological techniques to evaluate larval condition; and the recent discovery that larvae can be accurately aged using daily otolith increments. Some ideas for productive future research are proposed.

INTRODUCTION

Fishes are large and conspicuous members of marine communities. They have important commercial and recreational value, and their abundance can fluctuate widely in response to environmental variability or heavy exploitation. Fluctuations in abundance of fishes usually are caused by large annual differences in recruitment, which are related to mortality experienced by a cohort during the larval stage (28, 29). It has been difficult to evaluate potential factors that could affect recruitment in studies carried out on natural populations at sea because of the problem in estimating egg or larval abundances over extensive ocean areas, and because of an unpredictable environment whose effects cannot be controlled. During the past 15 years a capability has been developed by several laboratories to routinely culture marine fish larvae beyond embryonic and yolk-sac stages to the juvenile stage. Experiments on these laboratory cultured species has resulted in significant advances in our understanding of how environmental factors affect survival and growth of larvae.

Several papers recently have reviewed aspects of marine fish larvae culture (42, 50, 64, 65, 67). Although May's (67) evaluation of the critical period hypothesis, and Iwai's (50) review of feeding by fish larvae, included discussions of both laboratory and field-oriented studies, they did not specifically make conclusions about larval requirements based on experimental research. Blaxter's (14) general review of egg and larval development of fishes did summarize results of laboratory studies. We review some important results of recent experimental research on marine fish larvae and make conclusions about effects of environmental factors based on laboratory studies. Emphasis is on studies of species that have typical, pelagic larvae and includes the period from initiation of feeding until transformation to juvenile. Research on embryo and yolk-sac stages, aquaculture-oriented studies, and work on non-pelagic or non-typical larvae are not emphasized, although important contributions have been made in recent years.

Two major areas of research are reviewed and discussed. These are 1) the role of the food supply, the ability to feed, and the effects of predators; and 2) the role of physical and chemical variables, other than those due to man's impact on the environment. In addition, new techniques that hold promise for advancing environmental research on fish larvae are outlined and discussed.

FOOD REQUIREMENTS

The most important advance in larval culture technology during the past 15 years has been the determination of kinds and concentrations of living prey that give predictable survival and growth rates. The ability to undertake meaningful ecological, physiological and behavioral research developed once larvae could be routinely cultured. A myriad of foods has been used to rear marine fish larvae (64), but five foods have been more successful in recent years for meeting larval nutritional requirements. These are the rotifer *Brachionus plicatilis*, the nauplius of brine shrimp *Artemia salina*, copepods from wild plankton collections, the harpacticoid copepods *Tisbe* and *Tigriopus* spp., and the naked dinoflagellate *Gymnodinium splendens*.

Prey Concentrations

Marine fish larvae are visual feeders, with limited ability to search a volume of water for suitable food items during a unit of time. Suitable items usually are living organisms of a size that can be ingested, are nutritionally adequate, and are present at concentrations which allow a larva to encounter enough items during a day to meet its metabolic demands and to provide some excess for growth. Typical marine fish larvae are 2-3.5 mm long when they begin to search actively for food. Acceptable prey usually are 20-150 μm in breadth (7, 31, 56, 92). Some large and rather atypical larvae, like Atlantic herring, *Clupea harengus*, or plaice *Pleuronectes platessa*, can begin feeding on items in excess of 300 μm in breadth (10, 80, 82). Perhaps not surprisingly, required concentrations of prey for newly-feeding larvae have been shown to vary greatly in laboratory studies, the variation in large part reflecting size differences in the prey that has been offered.

Prey concentrations that have been used successfully to rear larvae have ranged from 1 x 10^1 to 2 x 10^5 per liter, although required concentrations for significant survival probably lie in the range 10^1 to 10^3 per liter. The highest reported concentrations (1-2 x 10^5 per liter) were of the large dinoflagellate *Gymnodinium splendens*, which can be used to culture northern anchovy larvae during the first week of life (47, 57, 95). Lowest concentrations (4-42 per liter) were of brine shrimp *Artemia salina* nauplii used to culture Atlantic herring larvae (82, 83). Neither *G. splendens* nor *A. salina* is usually available to marine fish larvae in nature, although Kiefer and Lasker (53) recently have shown that *G. splendens* may be present at 1-4 x 10^5 per liter in the chlorophyll maximum layer of the Southern California Bight. Northern anchovy larvae can concentrate in the chlorophyll maximum layer and can feed on *G. splendens* when its concentration exceeds 2 x 10^4 per liter (56). The most common prey reported from stomach analyses of marine fish larvae in nature are nauplii and other stages of copepods. Using copepod nauplii as food Houde (45) reported 10 percent survival at metamorphosis when per liter nauplii concentrations were 34 for sea bream *Archosargus rhomboidalis*, 107 for bay anchovy *Anchoa mitchilli*, and 130 for lined sole, *Achirus lineatus*. Other studies with wild plankton (predominantly copepod nauplii) as prey have reported higher concentrations required for significant survival than those reported by Houde (45). O'Connell and Raymond (73) estimated that more than 1000 nauplii per liter were required by northern anchovy larvae. Haddock *Melanogrammus aeglefinus*, larvae required 500-3000 per liter (58) and winter flounder, *Pseudopleuronectes americanus*, required 300-3000 per liter (60). It is possible that some reported prey concentrations required by larvae could be too high. Saksena and Houde (84) needed 1500-2000 nauplii per liter to successfully rear about 10 percent of bay anchovy larvae, but more recent experiments, with refined culture methods (45), have demonstrated that only 100 nauplii per liter are necessary to attain that survival rate. In some research, such as toxicological studies to determine effects of pollutants on larval survival, potential survival rates higher than 10 percent are required. For those studies, copepod nauplii concentrations of 1000 per liter or higher should be routinely employed (44, 45) (Table 13-1).

For six cases where copepod nauplii were fed to similar-sized larvae, the relationship between percent survival and nauplii concentration can be compared (Table 13-1). Haddock larvae had the highest required prey concentration, more than 2000 nauplii per liter being required for 10 percent survival. Winter flounder and northern anchovy larvae had an expected survival of 10 percent when nauplii were available at approximately 1600 and 1000 per liter, respectively. But, bay anchovy and lined sole required only about 100 nauplii per liter and sea bream needed less than 50 per liter to attain 10 percent survival. All of these species consume prey of similar types and sizes; the differences in requirements among species have not been explained. Temperature may play a role because the three species with lowest required prey concentrations were reared at 26-28°C, while the three with higher requirements were reared at 7-17°C. If searching ability and capture efficiency are enhanced at higher temperatures, required prey concentrations may decrease accordingly.

Rotifers, *Brachionus plicatilis*, are often used at high densities by aquaculturists to successfully rear fish larvae, but the minimum concentration required by larvae usually has not been determined. Hunter (46) estimated that 105 rotifers per liter were required by newly-feeding northern anchovy larvae to meet metabolic demands, a number that must be exceeded for larvae to grow. Lined sole larvae required from 60-120 rotifers per liter for 10 percent survival to metamorphosis (Houde, unpublished data).

Concentrations of microzooplankton in marine waters are not frequently reported, but when suitable collection techniques have been used observed, concentrations often are in the ranges of required prey densities determined in the laboratory (Table 13-2). Concentrations of suitable prey are exceptionally low in oceanic waters compared to coastal waters, and larval survival may depend upon the occurrence of relatively dense prey patches in oceanic areas.

Table 13-1. Copepod Nauplii Concentrations Used as Prey to Rear Six Species of Marine Fish Larvae and Corresponding Percent Survivals.

Reference	Species	Stage to which larvae were reared	Temperature (°C)	Nauplii Concentration (no./1)	Percent Survival
O'Connell and Raymond (1970)	Northern anchovy (*Engraulis mordax*)	12 days after hatching	17	10	0.0
				100	0.5
				1000	12.0
				4000	56.0
				8000	25.0
				14000	30.0
Laurence (1974)	Haddock (*Melanogrammus aeglefinus*)	metamorphosis	7	10	0.0
				100	0.0
				500	1.1
				1000	7.9
				3000	13.9
Laurence (1977)	Winter flounder (*Pseudopleuronectes americanus*)	metamorphosis	8	10	0.0
				100	0.0
				500	2.6
				1000	3.8
				3000	34.2
Houde (in press)	Bay anchovy (*Anchoa mitchilli*)	metamorphosing	26	50	11.6
				100	4.7
				1000	48.2
				5000	63.9
Houde (in press)	Sea bream (*Archosargus rhomboidalis*)	metamorphosis	26	10	3.9
				25	7.3
				50	12.7
				100	37.7
				500	72.4
Houde (in press)	Lined sole (*Achirus lineatus*)	nearly metamorphosed	28	50	1.4
				100	13.3
				1000	54.3

Table 13-2. Reported Concentrations of Some Microzooplankton Suitable as Prey for Marine Fish Larvae from Coastal and Estuarine Areas.

Reference	Place	Organisms	Concentration
Burdick (1969, cited in May, 1974)	Kaneohe Bay, Hawaii	copepod nauplii	50-100/1 common 200/1 sometimes present
Duka (1969)	Sea of Azov	*Acartia clausi* nauplii	62-65/1
		Other copepod nauplii and copepodids	> 30/1
		TOTAL	> 90/1
Mikhman (1969)	Gulf of Taganrog, Sea of Azov	early stages of copepoda	39-546/1
Hargrave and Geen (1970)	two eastern Canada estuaries	copepod nauplii and copepodids	> 60/1
Reeve and Cosper (1973)	Card Sound, South Florida	copepod stages 20-200 m in breadth	range 23-209/1 mean for 28 collections 72/1
		Tintinnids	range 40-369/1
Heinle and Flemer (1975)	Patuxent River estuary	*Eurytemora affinis* nauplii and copepodids	> 100/1 frequently > 2000/1 occasionally
Houde (unpublished data)	Biscayne Bay, South Florida	copepod nauplii and copepodids < 100 μm in breadth	usually 50-100/1
		Tintinnids	frequently > 100/1

Even in rich coastal waters, daily variability in microzooplankton concentrations occurs over order of magnitude ranges. Laboratory studies have shown that larvae deprived of food pass a "point of no return," after which they cannot initiate feeding (21, 67). This point can occur at only 0.5-2.5 days after yolk absorption for species at 20-32° (43, 57). Thus, unstable conditions that lead to temporary low prey concentrations probably are an important cause of mortality, even in areas where mean prey levels are high enough to sustain larvae.

Growth of larvae in relation to prey concentration can be determined in the laboratory. There are, of course, factors other than density of prey which influence larval growth. The size of prey, their caloric value, their percentage protein, and their digestibility are important. The effect of temperature makes it difficult to compare growth among species of larvae, even when similar foods have been used. Despite limitations in the comparative approach, larval growth responses to changes in food concentration can be demonstrated in the laboratory, and results extended to explain how densities of prey influence growth of wild populations.

A relationship between size at 16 days after hatching and copepod nauplii concentration was demonstrated for larvae of bay anchovy, lined sole, and sea bream (44, 45). Lengths and mean dry weights of survivors increased rapidly when prey level was raised from approximately 50 to 500 nauplii per liter. Lengths and weights tended toward asymptotes at food levels higher than 1000 per liter, although significant, additional growth could be obtained at higher prey levels. Laurence's data (58) on haddock larvae at six weeks of age show a similar relationship for prey concentrations in the range 500-3000 copepod nauplii per liter. Weights of winter flounder at 7 weeks of age in relation to copepod nauplii concentration also approached an asymptote at 1000 per liter prey level (60). O'Connell and Raymond (73) also found this type of relationship between length of northern anchovy larvae at 12 days and copepod nauplii concentration, except that prey ranged from 1000-14,000 nauplii per liter and the asymptotic size was not attained until prey level was approximately 8000 nauplii per liter.

Specific growth rates of marine fish larvae relative to prey concentration have been obtained in only a few instances. Specific growth rates (in dry weight) of haddock larvae were 7 percent, 8 percent, and 9 percent per day at copepod nauplii concentrations of 500, 1000 and 3000 per liter (58). The rates for winter flounder larvae, at the same nauplii concentrations were similar, 6 percent, 8 percent and 9 percent (60). Temperature for haddock experiments was 7°C and for winter flounder it was 8°C. Specific growth rates of sea bream and bay anchovy larvae at 26°, and lined sole larvae at 28°C can be estimated from Houde's data (45). The rates were 16, 20, and 28 percent per day for sea bream at 50, 100 and 500 per liter copepod nauplii concentrations; they were 16, 17 and 25 percent per day for bay anchovy at 50, 100 and 1000 per liter nauplii concentrations; but, lower rates of 7, 9 and 17 percent were obtained for lined soles at 50, 100 and 1000 per liter nauplii concentrations. Depending on prey concentration, the length of the larval stage can be highly variable. In the case of sea bream, specific growth rates at 100 and 50 per liter nauplii concentrations indicate that duration of the larval stage at those prey levels could be 1.4 to 1.7 times as long as at the 500 per liter level. Even if starvation was not a direct cause of mortality at low prey levels, the indirect effects of increased time of exposure to predators and possible enviornmental stresses during the larval stage, must have important consequences on the numbers that eventually metamorphose.

The density of prey, expressed as numbers per liter, provides a useful measure of availability of prey for capture by larvae, but does not necessarily provide a measure of energy available for growth and metabolism. Energy available is a function of prey density, prey size, and the ability of larvae to ingest particular prey, which is related to mouth size in many instances (10, 90). The kinds of prey also could influence the availability of energy, either through differential ability of prey to escape capture by larvae, or through differences in caloric content of prey. Few studies concerned with marine fish larvae have taken a bioenergetic approach to examine nutritional requirements. Such studies can provide the means to estimate amounts of ingested energy used for growth and metabolism. Estimates of required food intake, specific ration, growth efficiency and the critical minimum prey level all can be determined on a caloric basis using this method. When used in conjunction with studies on feeding by larvae in relative to prey concentration, valuable insight into nutritional requirements and feeding dynamics can be obtained. Recent work by Laurence (60) on winter flounder larvae is the best example of the use of a bioenergetic model for marine fish larvae.

The winter flounder larvae model (60) predicted critical food concentrations in the range 2.1-5.7 cal per liter, corresponding to 300-800 copepod nauplii per liter. Highest prey concentrations were required by newly-feeding larvae, suggesting that food was most critical at that time. Smallest larvae required most of the daylight period to obtain a minimum ration. Relatively high metabolic energy demands were made by the smallest larvae, reflecting their low efficiency in capturing food. Metabolic demands were lowest at high prey concentrations because larvae expended less energy in searching when food was readily available. Thus, for winter flounder larvae it appears that food consumption needs to be higher at low prey concentrations than at high prey concentrations. Estimated minimum consumption ranged from 18-230 nauplii per day over a range of larval dry weights from 10-1000 μg. Specific rations (μg consumed per μg larva x 100) decreased from nearly

300 percent for newly-feeding larvae to 27-31 percent for the smallest larval stages (10-75 μg), and continued to increase slowly for older larvae, ranging from about 18-33 percent for metamorphosed individuals. Laurence (60) predicted a continuous decrease in growth efficiency as prey concentrations were decreased, but so few values of growth efficiency are available for fish larvae that it is not possible to say whether this relationship will hold for other species.

Some valuable insight into feeding by marine fish larvae recently has been gained by combining results of bioenergetic studies on larvae with studies on feeding behavior and feeding ability of larvae. Blaxter and Staines (23) estimated swimming ability and feeding efficiency of herring, plaice, pilchard *Sardina pilchardus*, and sole *Solea solea* larvae. From their estimates they calculated the volume of water that could be effectively searched by larvae when they initiated feeding, and at sizes up to metamorphosis. Because swimming distances and volumes searched per unit time increased rapidly as larvae grew, larvae presumably needed higher prey concentrations during the youngest feeding stages. A similar approach was used by Rosenthal and Hempel (82), who in addition estimated the digestion time for herring larvae. They were then able to calculate the daily ration and required densities of prey (*Artemia* nauplii) for herring larvae at the end of the yolk-sac stage (10-11 mm) and at 13-14 mm length. Estimated ration was 40 *Artemia* nauplii per day at 10-11 mm and 50 per day at 13-14 mm. Required *Artemia* concentrations for larvae to obtain the rations at each of those length-classes were 4 to 42, and 2 to 25 per liter, respectively. Hunter (46) further extended the method by incorporating metabolic demands of larvae, and caloric values of prey (the rotifer *Brachionus plicatilis* and the dinoflagellate *Gymnodinium splendens*) into the prediction of food requirements. He concluded that first feeding northern anchovy larvae required 105 rotifers per liter or their caloric equivalents (e.g. 1785 *Gymnodinium* per liter) to just meet metabolic demands. Larvae at 10 days (5.9 mm) required only 34 rotifers per liter. In all of the examples, the relatively poor swimming ability and the low prey capture efficiency of first feeding larvae were demonstrated. This implies, as did Laurence's study (60), that food concentration is most critical at the first feeding stage and, when low, could be a significant cause of larval mortality in the sea.

It is possible to make many conclusions about larval food requirements based on dry weights of larvae, dry weights of prey, prey selection by larvae, digestion time, and estimates of the caloric values of the prey (cal/g ash free). Using these methods, Stepien (92) showed how feeding rates, specific rations and growth efficiency of sea bream larvae varied in relation to larval age, and to temperature for a single prey concentration. At 1000 copepod nauplii per liter, feeding rates for first feeding larvae (2-3 days after hatching) varied from 7.2

nauplii/hr/larva at 23°C to 17.6 nauplii/hr/larva at 29°C. These rates increased exponentially as larvae grew, so that larvae were consuming 53.8 nauplii/hr/larva at 23°C and 142.7 nauplii/hr/larva at 29°C at 16 days of age. Rations, in terms of numbers of nauplii and dry weight consumption, were then calculated. Specific ration also was calculated, and it tended to decrease as larvae grew, particularly at the highest temperature (29°C), where it was 220.8 percent at two days after hatching but decreased to 79.7 percent at 8 days. This result is similar to that of Laurence (60) for winter flounder (8°C), where specific ration decreased from over 300 percent for the smallest larvae to about 30 percent for metamorphosed individuals. Mean gross growth efficiency of sea bream (92) varied from 23.9 to 30.6 percent, the highest value being obtained to the lowest temperature (23°C); there was no evidence that growth efficiency changed with age. Mean growth efficiencies were similar to those for winter flounder (60), except that first feeding winter flounder had low growth efficiencies, which increased rapidly during the first few days of active feeding. The relatively high growth efficiency of sea bream, when it begins to feed, suggests that food is less critical for it than for winter flounder at that stage, a suggestion supported by the relatively low required prey concentration for survival of sea bream larvae (45).

Starvation Criteria

Because starvation is suspected as a major cause of larval mortality, biochemical, histological, and behavioral criteria have been developed for some species to show changes that occur when the food supply is inadequate. These techniques eventually may be used to characterize starving larvae collected at sea. Biochemical methods also have been used to show how laboratory-reared larvae differ from wild larvae. When supported by morphometric data, biochemical criteria hold promise to evaluate how types and amounts of food affect larval condition.

The biochemical composition of laboratory-reared, larval Atlantic herring and plaice was studied by Ehrlich (32, 33). He found that water, triglyceride, carbohydrate, nitrogen, carbon and ash content varied as a percentage of body weight as larvae grew, In starving larvae both relative (percentage) and absolute changes in amounts of those substances were measured, the relative changes often being a better measure of starvation than the absolute changes. Percentage of water increased about four percent in starved larvae of both herring and plaice, while percentages of triglyceride, carbohydrate, and carbon decreased. Percent nitrogen decreased in starved plaice larvae but did not decrease in herring; absolute amounts of nitrogen decreased in both species. Ash percentage of both species increased rapidly during starvation. Ehrlich (32, 33) concluded that the "point of no return" was not defined by an abrupt change in the chemical composition at some point during starvation, but rather that a continual change in chemistry occurred until the larvae became moribund.

In a similar study Anraku and Azeta (6) compared cultured and wild larvae and juveniles of the sea bream *Chrysophrys major*. They did not examine larvae less than 10 mm length, but for large specimens the cultured individuals tended to have a lower percentage of water, higher percentages of carbon and hydrogen, and a percentage nitrogen that did not differ from wild specimens until 20 mm length, when percentage nitrogen decreased in cultured individuals. Differences in food of cultured and wild specimens were the probable cause of differences in body chemistry. Starved individuals of sea bream showed effects similar to those for herring and plaice – i.e. increased percentage water and decreased percentages of carbon, nitrogen and hydrogen.

Histological changes in laboratory-reared larvae are indicative of starvation. Recent studies indicate that these criteria could be used to recognize starving or poorly nourished larvae in the sea. Umeda and Ochiai (96) examined fed and starved yellowtail *Seriola quinqueradiata* larvae, Ehrlich *et al* (34) examined herring and plaice larvae, and O'Connell (72) examined northern anchovy larvae. In all of these studies there were some similar findings. Intestinal epithelial cells atrophied in starving larvae and the intestine degenerated. The liver also degenerated in yellowtail, plaice and northern anchovy. O'Connell (72) and Umeda and Ochiai (96) examined the pancreas and found that its condition was markedly deteriorated in starved anchovy and yellowtail larvae. O'Connell (72) examined several other histological characters and found that starved anchovy larvae also had separations of muscular fibers and little intermuscular tissue, as well as notochord shrinkage. Using a discriminant function analysis he was able to discriminate 90 percent of starving larvae from fed larvae when four or more good histological characters were used. Ehrlich *et al* (34) found that there were good morphological characters associated with the histological changes, especially in herring larvae where severe head shrinkage and gut shrinkage caused a decrease in the "pectoral angle", and an increase in the eye height to head height ratio. Histological criteria as indicators of impending starvation seem excellent. They are relatively time consuming compared to morphometric analyses, but perhaps are more effective to distinguish starvation effects.

The concentration of prey affects larval behavior. Wyatt (100) demonstrated that duration of plaice larvae activity (searching behavior) was inversely related to prey concentration, and that starving larvae increased their time spent searching for food. This behavior presumably is adaptive and increases the probability of encountering prey when it is scarce. Using vertical migration as an index of activity, Blaxter and Ehrlich (20) found that fewer herring and plaice larvae vertically migrated after periods of starvation, and that starved larvae tended to be neutrally buoyant because of a relative increase in body water. They speculated that under starvation conditions larvae would be relatively inactive, suspended in midwater, and thus more susceptible to plankton net sampling than well nourished larvae. Blaxter and Ehrlich's (20) results differ somewhat from Wyatt's (100), partly because of the different criteria used to define activity.

Behavior of northern anchovy larvae in dense patches of prey, *Gymnodinium splendens* and *Brachionus plicatilis*, was investigated by Hunter and Thomas (49). In dense patches larvae swam slower and covered smaller areas. Reversals in swimming direction occurred more frequently in patches of food than in non-patch situations. The evidence strongly suggested that northern anchovy larvae were able to maintain themselves in suitable patches of prey, and that such an adaptation would allow larvae to take advantage of prey patchiness in the sea.

PREDATION

Predation almost certainly is the greatest direct cause of mortality to marine fish larvae, but there have been few attempts to evaluate its impact in laboratory studies. The food supply of larval fishes and other environmental factors can modify the predation mortality experienced by a cohort. As Cushing (29) noted, larvae that have an adequate food supply grow fast and swim well. Thus, they presumably avoid predation by growing quickly through the larval phase, when they are most vulnerable to a variety of planktonic predators. Similarly, pollutants or toxicants that retard larval growth or modify behavior could lead indirectly to increased predation mortality.

Four recent laboratory investigations have examined the potential impact of predators on larvae. Three species of pontellid copepods could more than meet their metabolic requirements by preying upon yolk-sac larvae of northern anchovy (62). Predation in 3500 ml beakers increased as anchovy larvae concentration was raised. Two of the copepods, *Labidocera jollae* and *L. trispinosa*, were only efficient as predators on yolk-sac larvae, but a third species, *Pontellopsis occidentalis*, also was able to prey on more developed, faster swimming larvae. The presence of alternate prey (*Artemia salina* nauplii) reduced larval mortality caused by *Labidocera* spp. in direct proportion to the numbers of *Artemia* that were present. In similar experiments, but using the euphausiid *Euphausia pacifica*, as an anchovy larva predator, Theilacker and Lasker (94) demonstrated that larval, juvenile and adult stages of the euphausiid could meet their daily carbon requirements by preying upon northern anchovy yolk-sac larvae. There was no strong evidence that anchovy concentration, when above 10 per 3500 ml, or the presence of alternate prey (*Artemia* nauplii) significantly influenced the predation rate on anchovies, at least by juvenile euphausiids. Euphausiids, like pontellid copepods, were most successful at capturing yolk-sac larvae. Based on laboratory results and known abundances of pontellid copepods, euphausiids, and yolk-sac anchovy larvae in surface waters off the coast of California, it is possible that copepods and euphausiids can have a significant influence on anchovy larvae survival (62, 94).

Yolk-sac larvae of the Pacific herring *Clupea harengus pallasi* were offered as prey to the amphipod *Hyperoche medusarum* in experiments conducted in 500 ml beakers (99). The numbers of larvae preyed upon increased as both predator and larval densities increased. But, the number of prey attacked per hour per predator decreased as predator abundance increased. The number of herring larvae attacked per hour increased, but the rate of increased slowed as the concentration of herring larvae was raised. When "flatfish" larvae were provided as alternate prey, the amphipods showed a preference for herring. Amphipods such as *Hyperoche medusarum* may be an important source of mortality to Pacific herring larvae in the sea, especially when the newly-hatched larvae are concentrated near the spawning areas.

Kuhlmann (54) investigated the chaetognaths *Sagitta setosa* and *S. elegans* and their possible role as predators on several species of fish larvae. Despite the often observed phenomenon in plankton samples of larval fish in chaetognath guts, *S. setosa* and *S. elegans* did not prefer the fish larvae in laboratory experiments when copepod prey was present in sufficient quantity. Kuhlmann (54) did not believe that the chaetognaths were important predators on larval fishes. He did find that both *S. setosa* and *S. elegans* consistently ate fish larvae after starvation periods of 24 to 48 hours if copepods were not offered as alternate prey.

ROLE OF NATURAL PHYSICAL AND CHEMICAL VARIABLES

A review of literature dealing with effects of natural environmental factors on development of marine fishes reveals a wealth of information on egg and yolk-sac stages (e.g., 14, 36, 38). However, these factors have not been intensively studied for larval stages from the time of first feeding to transition to the juvenile.

Light

Blaxter (16, 18) discussed the preferences of fish for light of specific intensities. This preferendum may vary from day to night and may not be available at the preferred intensity in some shallow water situations where little vertical migration is possible. The evidence reviewed by Blaxter (16) demonstrates that the light preferendum is variable among species and also among individuals.

Marine fish larvae typically are visual feeders and require a minimal light intensity above 10^{-2}-10^{0} lux to feed optimally (14, 16). Light levels reported by aquaculturists for successful culture of marine fish larvae have ranged from 250-10,000 lux (9, 42). A 500-3000 lux range has been used most often. A minimum light intensity is necessary for initial detection of prey, visual recognition and prey selection. At light intensities close to the threshold level there is a gradual reduction in larval activity and in feeding performance (16). Blaxter (19) recently has summarized information on the anatomy of eyes in fish larvae and also has discussed the development of vision. A pure cone retina, which requires relatively high light intensities to be effective, appears to be typical of larval stages of fish and such retinas have been identified in the Atlantic herring (22), the plaice (12) and other species (19, 23). In general, for the few species of larvae that have been studied, the light intensity range in which feeding activity decreases is approximately 10^{1}-10^{-1} lux (11, 12, 13). This is near the dusk-dawn light intensity range of 10^{2}-10^{-1} lux (16).

Laurence (60) estimated the number of hours required daily by winter flounder larvae at 8°C to consume a ration that exceeds the maintenance ration. For a prey concentration of 3.4 cal/1 (= 500 copepod nauplii/1), the minimum suitable for survival and growth, first feeding winter flounder larvae would require about 20 hours per day to consume the maintenance ration. The diurnal light period, when light intensities are above 10^{2} lux, is most important at low prey concentrations. For prey concentrations exceeding 6.8 cal/1 (= 1000 nauplii/1) winter flounder larvae could meet their daily food requirements in less than 10 hours and at prey levels above 13.6 cal/1 (= 2000 nauplii/1) established feeders could meet requirements in only 5 hours. It is apparent that the seasonal variation in day length and light intensity are important elements in any model predicting survival of fish larvae, particularly in high latitudes where seasonal variation is greatest.

Possible harmful effects of natural sunlight on larvae are poorly known. Ultraviolet light near the sea surface may be deleterious to pelagic eggs (63). Some information on effects of ultraviolet light on pelagic embryos may apply to larval stages. Pommeranz (75) tested effects of ultraviolet light on plaice embryos, using an artificial UV intensity of 0.05 ly/min ($1 \text{ ly} = 1 \text{ cal/cm}^2$) at the water surface of 350 ml incubators with a 200 ml/min water exchange. Although results were not conclusive, lower percentages of embryos survived in 12 hours and 24 hour-exposed incubators than in control incubators which were in the dark. Pommeranz (75) also exposed plaice embryos to natural daylight, natural daylight with UV wavelengths filtered out, and natural daylight with long wave infrared above 1400 nm filtered out. Average light intensities in two experiments were 257 ly/day and 462 ly/day. Only the high intensity experiment caused high mortality of embryos (35 percent). Ultraviolet light was considered to be the lethal agent because corresponding mortalities were not observed in incubators where ultraviolet was filtered out.

Temperature

Brett (25) refers to temperature as a polymorphic environmental factor that may be a lethal agent, a controlling factor regulating metabolism and development, a limiting factor restricting activity and distribution, a masking factor interacting with other environmental factors, or a directing agent such as a thermal gradient. It appears that the major temperature effect on marine fish larvae is that of a controlling factor regulating metabolic and developmental rates. In turn, those rates can affect survival of larvae through their influence on establishment of exogenous feeding and regulation of food requirements (e.g. 43, 57, 59, 60). For clupeiform, perciform, and pleuronectiform larvae, a 6-10°C range has been reported in which culture attempts are most successful (8, 10, 43, 57, 59, 61), although some survival can be obtained over wider temperature ranges.

There are few temperature effect-metabolic rate studies on marine fish larvae. Laurence (59) examined growth and metabolism of feeding winter flounder larvae at 2°, 5° and 8°C. Larvae reared at 5° and 8°C were tested until metamorphosis and the specific growth rate at 8°C (10.1 percent/day) was significantly higher than that at 5°C (5.8 percent/day). The growth rate at 2°C (2.6 percent/day) was less than at 5°C but not significantly less. Metamorphosis took 49 days and 80 days at 8° and 5°C, respectively. At 2°C larvae did not survive more than six weeks after yolk absorption. Power functions describing oxygen consumption of winter flounder in relation to body weight had exponential coefficients lower than the expected theoretical value of 0.80 (0.49 for 8°C, 0.56 for 5°C, 0.54 for 2°C). When separate power functions were fitted for larvae and for metamorphosed juveniles, the exponential coefficients for larvae closely agreed with the theoretical 0.80 value for all three temperatures, but the coefficients for metamorphosed juveniles were lower.

Hoss *et al* (41) examined the effect of a rapid 12°C rise in temperature (thermal shock) on growth of pinfish *Lagodon rhomboides* and spot *Leiostomus xanthurus*, and oxygen consumption of pinfish, to determine if growth and metabolism could be used to detect sublethal effects of power plant thermal pollution. The fish that they used were transformed juveniles, in most respects (5.15-7.89 mg dry weight for pinfish, 11.23-23.70 mg for spot). No significant difference in growth was observed for thermally shocked and control groups. Oxygen consumption rates of experimental and control pinfish indicated that a 12°C shock produced a slight increase in consumption rate which returned to normal levels within a few hours. Their determinations (41) of critical thermal maxima and survival after acute thermal shocks may not represent responses that might be obtained for smaller larvae. Time-temperature exposure histories are critical for determining thermal effects of entrainment (87, 89), but there are no such studies that include marine fish larvae from first feeding to metamorphosis stages.

During the past 15 years temperature responses often have been investigated in conjunction with effects of other environmental factors, usually variations in salinity for embryos and yolk-sac larvae (e.g. 66, 86). Multi-dimensional analysis has led to use of response surface models which permit evaluation of interacting effects such as between temperature, salinity, oxygen, and dose time (1). However, most of this research has dealt with the egg, embryo, and pre-feeding larval stages (e.g. 2, 3, 4, 5, 68).

Salinity

The developing eggs and yolk-sac larvae of many marine teleosts are known to tolerate wider ranges of salinity than they are likely to encounter under natural conditions (e.g. 2, 5, 36, 38, 68, 77, 86), but there are few studies dealing with salinity tolerances of typical pelagic marine fish larvae during the actively feeding stages.

In unaltered environments, the effect of changes in salinity on larval survival may be minimal, since pelagic larvae usually will be retained within a water mass that does not undergo extreme salinity changes. In the lower latitudes, where time for larval development to metamorphosis is short, the probability of an extreme salinity change that might cause mortality seems even less probable than in higher latitudes. Holliday (36), in reviewing data on salinity tolerances of Atlantic herring and plaice, observed that newly hatched larvae had a wider tolerance range for salinity than did metamorphosed juveniles. Tolerance to high salinities decreased from about 60^{o}/oo at hatching to about 40^{o}/oo after metamorphosis, while low salinity tolerance changed little during development, ranging from about 2-8^{o}/oo for both species. Kurata (55) obtained similar results for Pacific herring, *C. harengus pallasi* larvae which could tolerate a salinity range of approximately 2-60^{o}/oo at 10 days after hatching, but only 2-42^{o}/oo at 20 days.

There are several investigations on salinity tolerances of non-typical or non-pelagic marine fish larvae, from which conclusions about tolerances of marine fish larvae in general perhaps can be inferred. For mummichogs *Fundulus heteroclitus* the range of salinity tolerance was very wide, 0.39-100.00^{o}/oo (51). California killifish larvae *F. parvipinnis* also had a wide salinity tolerance, but the tolerance for low salinities decreased with age (76). Two atherinids, the California grunion *Leuresthes tenuis* and the Gulf grunion *L. sardina*, were tested for salinity tolerances during the larval stage (78, 79). Gulf grunion had a wider salinity tolerance range than did California grunion, but in both species the tolerance range decreased with age. A reasonable conclusion, based on limited data, is that newly hatched larvae of marine fishes are unlikely to suffer mortality as a direct effect of salintiy, but that older larvae are more vulnerable and could be killed by physiological stresses caused by salinity extremes.

Oxygen uptake of anesthetized Atlantic herring eggs and newly hatched larvae did not differ significantly at test salinities of 5, 15, 35 and 50°/oo (38). For larvae there was variable oxygen uptake, the rates sometimes being 10X the pre-transfer oxygen consumption rate. For example, for a transfer from 35 to 5°/oo at 8°C, larval oxygen consumption went from about 0.07 μl O_2/larva/hour to as high as 0.7 μl O_2/larva/hour within one hour after transfer. Oxygen uptake then fluctuated before returning to normal about five hours after transfer to the test salinity. Such fluctuations occurred for six-eight hours following transfer and were believed caused by osmoregulatory imbalance prior to acclimation to the treatment salinity.

Dissolved Oxygen

Vernberg (97) remarked that effects of low oxygen levels on animals are not easily determined under natural conditions because anoxic situations are always accompanied by other factors such as increased carbon dioxide and hydrogen sulfide concentrations. The effects of temperature and salinity on the solubility of oxygen also complicate the analysis of direct oxygen effect.

Dissolved oxygen requirements of developing eggs and larvae of Salmonidae and other freshwater or estuarine species have been investigated many times (e.g. 35, 91, 98). There are few studies on marine fish larvae to determine their tolerances to low oxygen tensions (30, 85). De Silva and Tytler (30) found that the incipient lethal oxygen level (LD_{50}) for Atlantic herring and plaice varied with development from the yolk-sac stage to metamorphosis. At 10°C the LD_{50} for yolk-sac larvae was 1.93 ml/1 for herring and 2.73 ml/1 for plaice. After larvae had been feeding for two weeks the LD_{50} was 3.08 and 2.66 ml/l respectively. At 56-63 days after hatching for herring and 42-49 days after hatching for plaice, gills developed and the LD_{50} levels fell to 2.91 and 2.52 ml/1, respectively. At metamorphosis, 70-80 days after hatching for herring and 77-84 days after hatching for plaice, the LD_{50} was 2.17 and 1.69 ml/1, respectively. De Silva and Tytler (30) also measured routine metabolism of herring larvae from 7-62 days after hatching and plaice larvae from 5-75 days after hatching at 10°C. For the relationship between oxygen consumption and weight, they obtained exponential coefficients of 0.82 for herring and 0.65 for plaice. These values are higher than the values 0.49-0.56 obtained by Laurence (59) for winter flounder from hatching through metamorphosis, although Laurence obtained a coefficient of 0.80 when he excluded metamorphosed individuals from his analysis.

In surface waters of the euphotic zone dissolved oxygen usually ranges from 4-8 ml/l with supersaturation (> 6-9 ml/l) possible in highly productive shallow coastal waters. Kalle (52) reported that in coastal areas with high primary production, oxygen super-saturations up to 120 percent are not unusual during periods of intensive solar illumination. In shallow waters, temporary super-saturations may approach 500 percent (52). Mortality or stress of fish larvae due to low oxygen tensions probably occurs only under unusual conditions in the sea.

Miscellaneous Environmental Factors

Environmental factors such as turbidity, mechanical stresses, and shear forces likely to be found in nature have not been studied experimentally with regard to effects on marine fish larvae. A few investigations of effects of these factors on embryonic stages indicate that embryos are resistant to high sediment suspensions and mechanical forces which are present in the environment (e.g. 71, 75, 88).

Schubel *et al* (88) observed that striped bass *Morone saxatilis* eggs could tolerate silt loads up to 500 mg/l. They noted that turbidity in areas being dredged could be as high as 1000 mg/l, which would cause significant embryo mortality, but that such high concentrations rarely occurred. Hoss *et al* (40) tested larvae of seven estuarine species with three concentrations of sediment extracts (the supernatent from 500 g of Charleston Harbor sediment shaken in one liter of filtered seawater). Under their laboratory conditions, survival of larval pinfish and menhaden *Brevoortia tyrannus* was 25-0 percent at the 75 and 100 percent test concentrations. The supernatent water and sediments were not analyzed for toxic substances by the authors, but they cited references to relatively high levels of lead, copper, zinc and chromium in Charleston Harbor sediments.

Pommeranz (75) investigated mechanical properties of plaice eggs by deforming them with a lever. The force to burst the chorion varied with time from fertilization and ranged from about 1.5 g during the 30 minutes after fertilization to a mean of about 700 g and 600 g for gastrula and embryo stages, respectively. For comparison purposes, Pommeranz (75) cited one rough estimate of the pressures developed by a spilling breaker in the open sea as approximately 0.1 kg/cm^2.

Morgan *et al* (71) subjected striped bass and white perch *Morone americana* embryos and yolk-sac larvae to experimental shear forces of 0-86 dynes/cm^2 over exposure times of 1-20 minutes. The estimated median lethal shear (LS_{50}) that could kill 50 percent of the embryos and larvae ranged from 120 dynes/cm^2 for a 20 minute exposure, to 785 dynes/cm^2 for a one minute exposure. Estimated LS_{50} values for striped bass yold-sac larvae were 785 dynes/cm^2 and 300 dynes/cm^2 for 1 and 4 minute exposures, respectively. White perch yolk-sac larvae were more vulnerable, their LS_{50} values were 415 dynes/cm^2 and 125 dynes/cm^2 for 1 and 4 minute exposures respectively. Calculated average shear force in the Chesapeake and Delaware Canal, where striped bass eggs occur, was only 13.8 dynes/cm^2, far below the estimated LS_{50} values. The authors (17) also related these LS_{50} values to expected shear forces of 72-230 dynes/cm^2 that might be present in the water box of a power plant cooling system. The 230 dynes/cm^2 shear approaches the 4 minute LS_{50} value for striped bass yolk-sac larvae and exceeds it for white perch.

Chipman (27) reviewed literature on effects of naturally occurring ionizing radiation on marine animals. He found no convincing evidence to demonstrate that marine animals showed any response, functional or structural, to ionizing radiation levels present in the environment. In marine animals observable effects are primarily at the cellular level, and the radiation tolerance is a function of the dose-rate, time patterns of exposure and metabolic rate; consequently, effects would be most evident during embryonic development (27).

FUTURE RESEARCH

Both laboratory and transitional laboratory-field studies will extend our knowledge of environmental effects on larval stages of marine fish. A recent colloquium on larval mortality and the recruitment problem has defined some areas in need of research (48). Emphasis of that colloquium was to advocate research related to starvation and predation, the two factors that probably have the greatest effect on recruitment of year classes. Environmental stresses from man's activities are additional threats, particularly to estuarine species or those found over the continental shelf. Pollution effects on embryos can cause gross functional and structural abnormalities that may produce yolk-sac larvae incapable of surviving to the exogenous feeding stage (81). Larvae can be equally vulnerable to deleterious effects of pollutants, and their responses to this stress may be reflected in impaired predator avoidance behavior and food capture efficiency. More subtle effects could involve functional disruptions of metabolism, temperature and salinity tolerance, and enzyme-substrate interactions. Both direct and indirect effects of environmental modification on recruitment need to be determined.

The ability to culture larvae widens the possibilities for laboratory research which will help interpret results of field studies. The larval stage is a dynamic one, characterized by fast growth, sometimes spectacular developmental changes, and frequent shifts in behavior. Typical toxicity bioassays, where times to 50 percent mortality are estimated, may not be the best approach to determine how environmental factors affect survival of a larval cohort. Environmental factors act in concert, and it is the sum of experiences over the entire embryo and larval stages that determines whether a good or poor year class results. A bioassay for 96 hours, testing one or two factors, usually can provide only a rough evaluation of the potential effect of the factor(s) on recruitment. More meaningful conclusions can be drawn from investigations that encompass the entire larval period. Many studies of that kind have been carried out on larvae of freshwater fishes (69), but the difficulties in rearing larvae of marine species have limited most bioassay research to embryo and yolk-sac larva stages.

Experiments in large volumes of seawater, either in plastic bag enclosures, such as those used in recent Controlled Ecosystems Pollution Experiments (CEPEX) (70) or in large tanks (74) hold great promise because whole communities can be entrapped in such volumes. Effects of predation and competition can be evaluated. Direct and indirect effects of added pollutants on each trophic level can be observed. Recruitment success or failure by fishes in such systems can be interpreted in the context of observed changes that took place in the plankton community during the course of larval development.

Other approaches include transitional studies that combine laboratory and field experiments. The "field bioassay" developed by Lasker (56) uses laboratory-reared larvae in shipboard experiments, in which larvae are reared in natural seawater sources to evaluate the potential of particular water masses to support larval survival and growth. The recent discovery, based on laboratory studies, that daily growth rings are present on otoliths of larvae, will allow better estimates of larval growth and mortality rates in the sea (26, 93), and also will allow comparison of growth in the laboratory with growth under natural conditions.

Except for swimming-feeding behavior of a few species and behavioral responses to varying light levels (12, 15, 17) little is known about normal behavior patterns of larvae or changes in behavior induced by environmental effects. Behavioral studies not only can increase our understanding of how pollutants affect larval behavior, but they also can provide important insight into how predation and competition operate during the larval stage.

There are many techniques presently available that allow environmental factors and their effects on marine fish larvae to be evaluated. In the next 10 years, culture of marine fishes will be routine procedure at many laboratories; and as more data accumulate, some of the seemingly contradictory results obtained to date, especially with regard to critical food concentrations, will be resolved. Additional species of marine fishes need to be tested for larval tolerances to environmental factors. Present day literature is dominated by research on herring, plaice, and northern anchovy; the first two species are rather atypical pelagic, marine fish larvae because of their unusually large size and advanced development at hatching. Refinement of culture methods, improved techniques for handling and testing delicate larvae, and examination of multiple factor effects and interactions throughout the period of larval development will help us to better understand how the environment acts on a cohort of larvae. This knowledge can be incorporated into predictions of recruitment success based on probable influences of environmental factors operating during the larval stage.

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LABORATORY CULTURE OF THE GRASS SHRIMP
Palaemonetes vulgaris

by

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ABSTRACT

Experiments have been undertaken to test the feasibility of hatching, rearing, and breeding an in-laboratory population of the grass shrimp, *Palaemonetes vulgaris*. Primary objectives include continual availability of all life stages (for use in experiments or as food organisms) and comparisons of lab-reared and field-collected animals.

Systems have been designed for culturing the grass shrimp throughout its life cycle. Larval survival percentages reached 70 percent in the beaker and "hatching jar" culture systems. Up to 75 percent of these metamorphosing larvae survived to adult stages. Both *Artemia salina* and the flake food Tetra Marin were proven to be successful diets for *P. vulgaris*.

Results indicate that *P. vulgaris* can be maintained and propagated in the laboratory. Larvae hatched in the lab have been induced to produce normal larvae within as little as 90 days. This generation time is apparently shorter than the time in field populations.

INTRODUCTION

Most marine biology research efforts require a consistent supply of experimental animals. Field-collected organisms often confer variability due to individual differences in life history, nutrition, etc. Many of these problems can be controlled by culturing the animals under rigorous, well-documented laboratory conditions.

The purpose of this study was to develop and standardize laboratory holding and culture techniques for the grass shrimp, *Palaemonetes vulgaris* (Say). Establishment of suitable methods would permit testing of the feasibility of hatching, rearing and breeding a laboratory population of the shrimp for use as experimental animals. Of primary consideration in this study was the development of flow-through culture systems for the various life stages. Static designs represent poor simulations of field conditions and may impose unnecessary stresses on the animals (8). A secondary concern was to determine suitable diets for the juvenile and adult grass shrimp. Broad (1) found brine shrimp, *Artemia salina*, nauplii to be a very satisfactory larval food. Success for the project must be measured in terms of growth, survival, and population reproduction.

The advantages of using lab-reared organisms are countered by several anomalous characteristics of organisms maintained in the lab. Morphological changes, as compared to field animals, have been noted by Paul Yevich (Environmental Research Lab, Narragansett, R.I.; personal communication) in many marine animals. However, the increased control of age, nutrition, and prior exposure to environmental variables would appear to outweigh slight changes in morphology.

The grass shrimp, *Palaemonetes vulgaris* (Say), was selected for these studies for several reasons: the shrimp is a common estaurine species available to researchers along the Atlantic and Gulf of Mexico coasts (9); the animal is relatively easy to rear in the laboratory; and the life cycle can be greatly compressed in the lab (4).

EXPERIMENTAL

Several ovigerous grass shrimp were collected by dip net on 14 July 1976 in the Pettaquamscut River estuary adjacent to Narragansett Bay, Rhode Island. Mid-summer salinities at the collection site range from 25-30o/oo depending on the tidal cycle; water temperature was 21.5°C.

Egg-bearing females were isolated in six ℓ (1.6 U.S. gal.) tubs[1] at 21.0-23.5°C and oceanic salinities (29-34.5o/oo). Photoperiod was maintained at ambient levels of L14:D10. Water was changed daily and aerated gently. Shrimp were offered food during this holding period but they rarely fed.

Larvae hatched after 1 to 17 days of holding and were immediately pipetted into the flow-through system shown in Figure 14-1. About 200 larvae were held in this two ℓ system for 21 days, by which time all shrimp had reached the late larval stages. Developing larvae were fed excess quantities of newly hatched and one-day old brine shrimp nauplii, *Artemia salina* (San Francisco Bay Brand).

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[1] Rubbermaid Commercial Products, Inc., Winchester, VA.

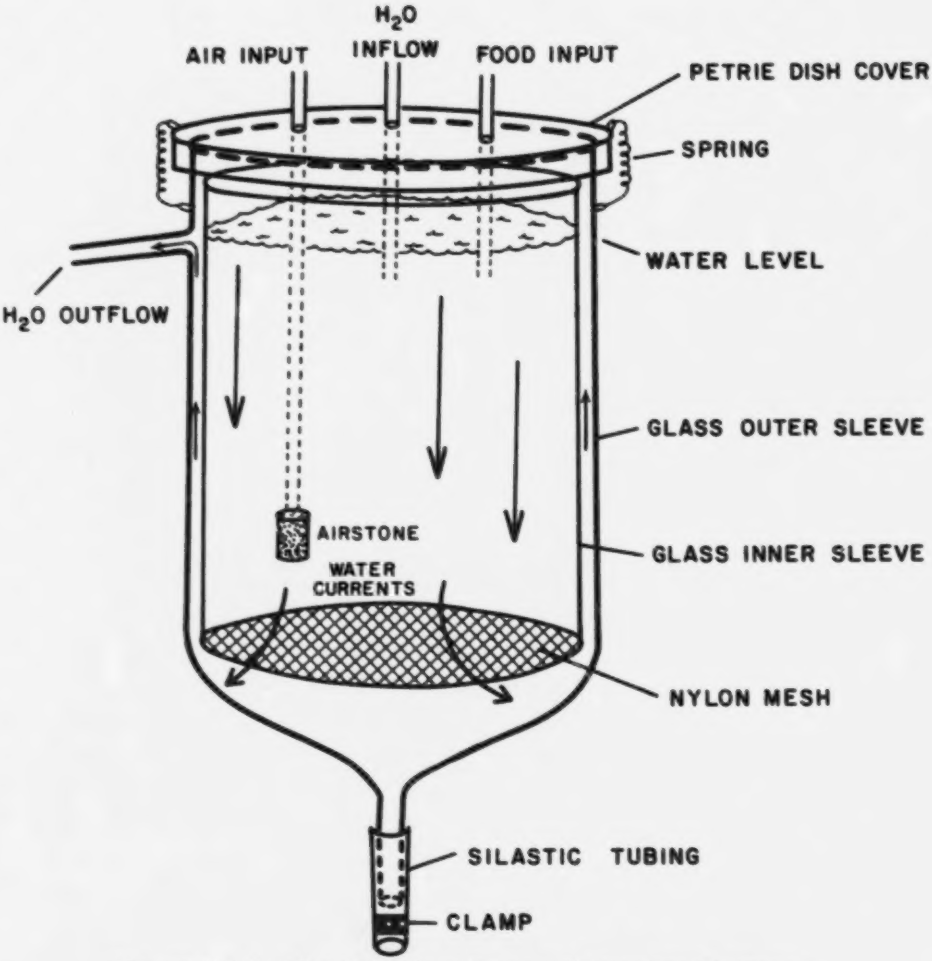
H_2O INFLOW
AIR INPUT
FOOD INPUT
PETRIE DISH COVER
SPRING
WATER LEVEL
H_2O OUTFLOW
GLASS OUTER SLEEVE
AIRSTONE
GLASS INNER SLEEVE
WATER CURRENTS
NYLON MESH
SILASTIC TUBING
CLAMP

Figure 14-1. The 2 ℓ Flow-Through Beaker System.

NOTE: Designed by Dr. W. B. Vernberg under E.P.A. Grant R 802071 and modified for use in the culture of larval and juvenile *Palaemonetes vulgaris*.

All surviving juvenile *P. vulgaris* (about 140) were transferred on day 22 (post-hatch) to the 20 ℓ "hatching jar"[2] commonly used in hatching and rearing larval fish (Figure 14-2). This 30 cm diameter, clear acrylic tank, was modified from the manufacturer's design by placing a 400 μm nylon mesh across the outflow ramp, thereby eliminating the need for a mesh over the top of the entire system. This smaller outflow area effectively decreased the chances of impinging larvae on the mesh. Gentle aeration and a water flow of 50 ml/min created a satisfactory circulation pattern. This hatching jar system

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[2] Midland Plastics Co., Brookfield, WI.

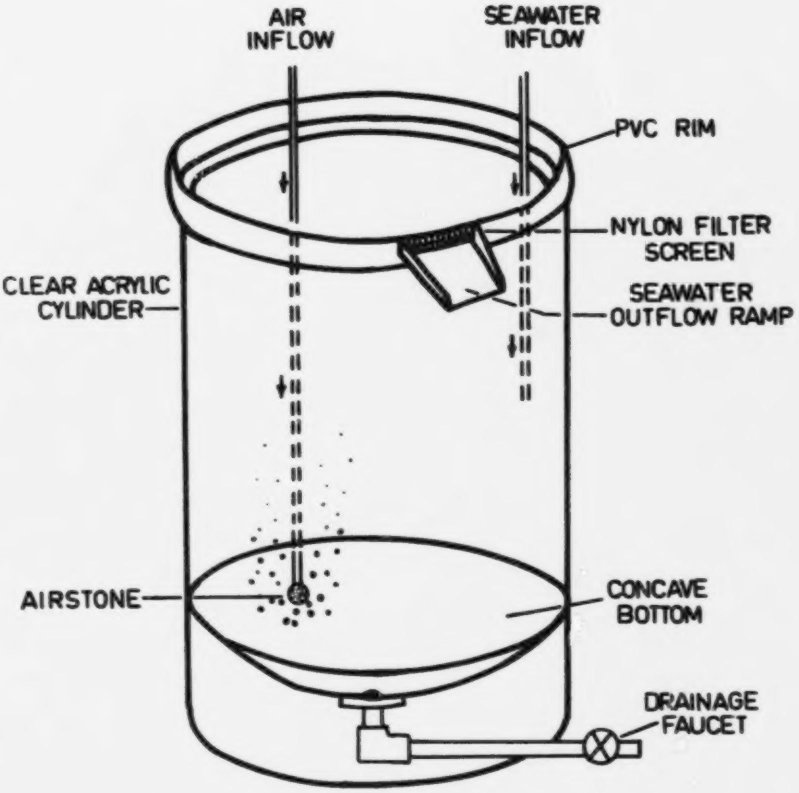
AIR INFLOW
SEAWATER INFLOW
PVC RIM
NYLON FILTER SCREEN
CLEAR ACRYLIC CYLINDER
SEAWATER OUTFLOW RAMP
AIRSTONE
CONCAVE BOTTOM
DRAINAGE FAUCET

Figure 14-2. The 12 ℓ Hatching Jar System Used to Culture Juvenile and Adult Grass Shrimp, Palaemonetes vulgaris.

NOTE: 20 and 48 ℓ sizes are identical in design.

was used for 14 weeks, during which time a combination of thawed and live juvenile brine shrimp were fed in excess, with unconsumed food removed by siphon once each week.

All grass shrimp were transferred from the 20 ℓ jar at age 17 weeks. Of these shrimp, 40 were used in a diet study, and the remaining 66 were placed in a 48 ℓ hatching jar, a scaled-up version of the 20 ℓ size (Figure 14-2). In the diet study, 40 shrimp were divided evenly between two 4 ℓ systems resembling that shown in Figure 14-1. One group of 20 shrimp was reared on lab-reared *Artemia* adults while the other group was fed 243–400 μm pieces of the commercial flake fish food, Tetra Marin. Animals were fed daily in excess. Growth in carapace length, survival, and the incidence of ovigerous females were considered in determining the suitability of each diet.

RESULTS AND DISCUSSION

Attempts to culture and maintain a laboratory population of *P. vulgaris* have been successful thus far. Ovigerous females were obtained from adults hatched in the laboratory and cultured for 90 days. These egg-bearing females have yielded morphologically normal larvae, thereby indicating that the eggs resulting from lab-reared females are viable. After 16 months of culture, a total of 21 ovigerous females have been collected from the system. Six of these shrimp are females that also bore eggs in the first laboratory spawning season (November, 1976 to January, 1977 or 9 to 10 months ago). Problems in controlling photoperiod and water temperature have limited successful hatches to only two females. Little (4) has discussed how manipulation of these two environmental factors can be used to induce winter breeding in grass shrimp.

The diet study (Table 14-1) has indicated on a gross scale that a flake food can be used as a diet. Ovigerous females were collected from the four ℓ flow-through systems used for both the brine shrimp and Tetra Marin diets. Therefore, growth, survival and reproduction are acheivable with the live and dried foods.

All of the systems and techniques mentioned herein have yielded satisfactory results. However, some minor problems remain. One such problem is cannibalism, especially in the 20 and 48 ℓ hatching jars used as holding tanks for juvenile and adult grass shrimp. Obvious solutions include increasing the food available, either as more food per day, or as multiple daily feedings, or decreasing the density of shrimp. A certain degree of cannibalism is to be expected in mass cultures during periods of molting.

A second problem, also in the hatching jar systems, relates to the physical design of the container. The concave bottom of the jar, coupled with a circular flow, causes a centrifuging of the shrimp into the center near the bottom. A flatter bottom with a larger bottom surface area to volume ratio could be a solution. The 40 ℓ kriesel systems (3) used in lobster culture efforts have the desired flatter bottoms, and also jetted water inflow along the sides that create a more uniform distribution of the shrimp. Preliminary studies indicate that the kriesel design will be very successful for juveniles and adults. Compared to growth in the four ℓ beaker systems (See Table 14-1), the kriesel has yielded significantly higher growth rates. Mean carapace length in the kriesel after nine weeks was 6.8 ± 0.69 mm (range 5.9 to 7.9 mm), a size not attained until an age of about 20 weeks in the beaker.

One last problem is animals flipping out of the systems, especially the 48 ℓ jar. This appears to happen in conjunction with a molt, and the subsequent cannibalism pressure from other shrimp in the system. A simple solution to the

Table 14-1. Summary of growth, measured via carapace length, and survival of juvenile and adult Palaemonetes vulgaris cultured in excess concentrations of live, adult Artemia and ground Tetra Marin.

		Artemia salina				*Tetra Marin*			
Date of Sampling	Age (Weeks)	Mean Length (mm)	S.D.	Range	No. of Survivors	Mean Length (mm)	S.D.	Range	No. of Survivors
11-6-76	14	6.4	± 0.65	5.1-7.4	20	6.5	± 0.66	5.4-7.7	20
12-14-76	20	-	-	-	19	-	-	-	18
2-7-77	28	7.5	± 1.05	6.2-10.4	18	8.0	± 0.96	5.4-9.9	18
5-23-77	45	-	-	-	17	-	-	-	15

NOTE: Initial counts of shrimp were 20 per four ℓ beaker. Carapace lengths were measured from tip of rostrum to posterior edge of carapace.

escape problem is a cover over the system. However, such a design may change the cause of mortality from escape and desiccation to cannibalism.

Each of these systems emphasizes low maintenance and unlimited scale-up potential. Care was taken during the design phase to avoid sharp corners, excess mesh area, or eddying currents. Hartman (2) has shown that brachyuran larvae become impinged in corners that break spines or setae and impede molting. He also mentioned the importance of tapered walls so that larvae and food do not become caught in eddying currents.

Even with these problems, survival of all life stages has been high. Larvae cultured in the beaker systems have shown approximately 70 percent survival when fed *Artemia* nauplii in excess. In this experiment, survival of juveniles and adults reared in the 20 ℓ hatching jar was 75 percent (since metamorphosis) over a 14 week period. The diet studies have shown similar survivals in smaller cultures.

Several other comments are worthy of mention. The ages at transfer from one system to another represent the schedule used in this study and most likely could be altered without problem. Also, one key factor to consider in the two ℓ and four ℓ flow-through systems and the hatching jars, is mesh size. A mesh should be chosen that will permit debris to pass through, yet retain both larvae and food organisms. For these reasons a 243 or 400 μm mesh was used.

Use of *A. salina* nauplii as a diet for larval grass shrimp has been substantiated by several investigations (1, 6). The study by Broad (1) confirmed that diets including brine shrimp were more successful in terms of survival and development than diets lacking this animal tissue. Provenzano and Goy (6) established the possibility of using *Artemia* from several locations, including San Francisco, Canada and, to a lesser degree, Utah.

Establishment of a laboratory population of grass shrimp should lead to increased use of the animal in bioassays. Nimmo *et al* (5), based on cadmium bioassays, concluded that adult *P. vulgaris* were "acutely and chronically" more sensitive than the pink shrimp, *Penaeus duorarum*. Studies by Shealy and Sandifer (7) have shown the susceptibility of *P. vulgaris* larvae to mercury. Conversely, Vernberg *et al* (8) reported that *P. pugio* is quite resistant to cadmium bioassays. Apparently animal age and species are important; *P. vulgaris* may prove to be a better pollution indicator than *P. pugio*.

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EVALUATION OF VARIOUS DIETS ON THE LIPID AND PROTEIN COMPOSITION OF EARLY LIFE STAGES OF THE ATLANTIC SILVERSIDE

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ABSTRACT

A study was performed to evaluate the effect of various natural and artificial diets on the lipid and protein composition of laboratory cultured Atlantic silversides, *Menidia menidia*. Results were compared to analyses of wild silversides, which constituted the biochemical control.

The best growth and survival of juvenile silversides was obtained on a live 3-day-old brine shrimp nauplii diet. Substantially lower growth and survival were obtained on a freeze-dried brine shrimp diet and the artificial diets.

Amino acids were incorporated into the tissue of batch cultured silversides fed a live 3-day-old brine shrimp diet by the fifth day of culture. Thereafter, the profiles changed very little, except for the levels of histidine and arginine in the 58-day-old silversides. The amino acids of the cultured fish fed natural or artificial diets were quite similar. Bioavailability studies are necessary to ascertain the degree of incorporation and assimilation of dietary amino acids.

The whole body fatty acid composition of cultured fish reflected the composition of their diets. Fish fed a live brine shrimp nauplii diet had higher total lipid levels and lower polyunsaturated fatty acid levels than wild silversides. Cultured fish may store large amounts of lipids in order to facilitate the bioaccumulation of long chain polyunsaturated fatty acids. The incorporation of cod liver oil into a diet previously containing a soybean oil increased the levels of the polyunsaturated fatty acids in the fish. The resulting fatty acid tissue levels resembled the long chain fatty acids of the wild fish lipids more closely than the profiles of fish fed brine shrimp nauplii.

INTRODUCTION

The Atlantic silverside, *Menidia menidia*, is a marine fish used in bioassay studies due to its relative sensitivity to environmental contaminants (17). Bioassays using silversides have been relatively short-term studies, principally because of the dependence upon wild fish populations. Before long-term studies are possible, the dietary aspects of laboratory culture technology must be developed. The diet can affect the organism's ability to respond in a reproducible fashion. Additionally, the diet is an important feature in the ability of cultured fish to reach maturity, and spawn viable eggs necessary for multi-generation bioassay evaluations.

Live brine shrimp, *Artemia salina*, have been used world-wide in the laboratory culture of larval marine fish (4). Silversides used in toxicological bioassays by the Environmental Protection Agency's Environmental Research Laboratories have commonly been fed brine shrimp as their primary diet. However, the difficulty of culturing large volumes of biochemically similar brine shrimp (8), coupled with increased costs and decreased availability (32) of cysts has mandated the need for an artificial diet. Providing an adequate, practical, and economical diet is a major factor limiting culture of most marine fish reared on either a laboratory or commercial scale. Based on these facts, the University of Rhode Island, Food Science & Technology, Nutrition and Dietetics Department collaborated with the Environmental Research Laboratory to evaluate a number of artificial diets that could replace brine shrimp.

In our study, we were attempting to produce a cultured fish that could respond in bioassays in a similar manner to wild fish, and provide comparable growth and survival as brine shrimp fed juvenile fish. This paper discusses the effects of various diets on the protein and lipid composition of laboratory reared silversides.

EXPERIMENTAL

General

This study consisted of three parts: 1) a two month batch culture of silversides fed 3-day-old brine shrimp, 2) a preliminary evaluation of an artificial Atlantic salmon, *Salmo salar*, diet comprised of a soybean oil base and 3) an expanded study using brine shrimp and a number of artificial diets.

Culture

The collection of the gravid female silversides, the stripping and fertilization of the eggs, the hatching and feeding procedures, and the culture systems used in this study have been previously documented (7). Gravid fish were collected from Bissel Cove, Narragansett Bay (R.I.) and transported in aerated containers to holding tanks located at the Environmental Protection Agency Laboratory, Narragansett, R.I. Eggs were stripped from females onto nylon monofilament screens with a mesh size of 400μ, and fertilized by bathing them in the milt of two to three males (5). They were then suspended in egg hatching jars, 15 cm in diameter, modified from the original design of Buss (12) by the addition of a bottom center drain.

After hatching, the fish were transferred to a 720 liter holding tank and fed live 3-day-old brine shrimp. Fish were periodically removed during the two month batch culture study for biochemical analyses. Fish used in both the preliminary and expanded diet evaluations were cultured for approximately two weeks. The jars used for hatching of the eggs, were also used as the culture vessels in the artificial diet studies. For the eight-diet expanded study each jar was stocked with 50, 23-day-old fish (individual mean weights, 8.90 mg) obtained from the batch culture population. Two replicates were run for each diet fed group.

Diets and Feeding Procedures

The wild plankton (Diet 1) were collected from a number of locations in the west passage of Narragansett Bay, R.I. and from local estuarine areas with a 243μ mesh conical plankton net (Table 15-1). The plankton population was comprised of a mixture of copepods, primarily *Acartia tonsa*, and some invertebrate larvae (22). The plankton samples were transported to the laboratory in insulated containers and held at 20°C.

The live brine shrimp nauplii (Diet 2) (San Francisco Bay Brand, USA) were incubated for 24 hours in two liter separatory funnels containing filtered seawater (29.0 to 31.0 o/oo salinity, 20 to 22°C) and harvested after 72 hours. A starved group served as a control (Diet 3). The freeze-dried brine shrimp (Diet 4) was obtained by freezing the live brine shrimp to -38°C and then drying at 4μ/Hg pressure for 24 hours.

Diets 5 through 9 were the artificial formulations. Tetra Marin (Diet 5) is a commercial flake diet used in aquarium fish applications and consists of unknown proportions of meals from fish, crab, mussel, lobster, beef heart, and brine shrimp. In addition, it is made up of such components as halibut liver, *Calanus finmarchicus*, kelp, oatflour, wheat germ, *Agar-Agar*, seaweed, and bone charcoal. The other four artificial diets were modified formulations originally prepared to suit the requirements of Atlantic salmon. The diets were prepared by the Tunison Laboratory of Fish Nutrition, U.S.F.W.S., Cortland, New York. The composition of these diets is given in Tables 15-2 and 15-3.

Table 15-1. Description of the Experimental Diets

Experimental Diets	Source and Description[1]
1. Wild plankton	Collected in West Passage, Narragansett Bay by conical net with 243μ mesh opening and retained on a 116μ mesh sieve.
2. Brine shrimp nauplii, live	San Francisco Bay brine shrimp, *Artemia salina*, eggs hatched and harvested after 72 hours in filtered and autoclaved seawater of 20-22°C and 20-30 o/oo salinity.
3. Starved	Unfed
4. Brine shrimp nauplii, freeze-dried	Nauplii as obtained in diet #2, then freeze-dried 24 hours to constant weight.
5. Tetra Marin	Lot #125244. Tetra Marin Staple Food, Tetra Werke Dr. rer. nat. Baensch, Melle, West Germany
6. Artificial, CM-1	Cortland #1 diet with cod liver oil. (See Table 15-2)
7. Artificial, C-1	Cortland #1 diet with soybean oil. (See Table 15-2)
8. Artificial, CMP-1	Semi-purified diet with cod liver oil. (See Table 15-3)
9. Artificial, CMP-2	Semi-purified diet with cod liver oil and an amino acid supplement. (See Table 15-3)

[1] All diets were ground to a coarse powder of 400μ size or less.

Fish were fed four times daily at a level of five percent body wet weight. A compensation factor was provided to accommodate the flushing action of the system (7).

Protein Analysis

All fish were starved for 24 hours prior to sacrificing to reduce the stomach contents. The diet and fish samples were acid hydrolyzed according to the

Table 15-2. Composition of the Artificial Diets, CM-1 and C-1

Components	Percent Composition CM-1	 C-1
Herring meal	40.00	40.00
Soybean oil	10.00	10.00
Corn gluten meal, 60%	10.00	10.00
Wheat middlings, standard	9.00	9.00
Brewers dried yeast	5.00	5.00
Dried condensed fish solubles	5.00	5.00
Dried Whey	5.00	5.00
Meat and Bone Meal	5.00	5.00
Soybean oil	–	10.00
Cod liver oil	10.00	–
Mineral mixture[1]	0.40	0.40
Vitamin mixture[2]	0.70	0.70

[1] Mixture provided the following compounds in g/kg diet: $MgSO_4$, 2.0; $ZnSO_4$-H_2O, 0.3; $FeSO_4$-$7H_2O$, 0.3; $CuSO_4$, 0.3; KIO_3, 0.0091 and $MnSO_4$-H_2O, 1.0.

[2] Mixture provided 10,000 IU Vitamin A as retinyl palmitate; 4,000 IU Vitamin D as Cholecalciferol; 75 IU Vitamin E as dl-α-tocopheryl acetate; and the following amounts (milligrams) of other vitamins per kilogram of diet: menadione dimethylpyriminidol bisulfate, 10.0; thiamine HCL, 4.0; riboflavin, 30.0; calcium pantothenate, 150.0; niacinamide, 300.0; pyridoxine-HCL, 20.0; d-biotin, 6.0; folacin, 15.0; Vitamin B_{12}, 0.002; L-ascorbic acid, 1000; inositol, 500.0; butylated hydroxytoluene (100%), 100.0; and choline chloride (70%), 1330.0.

methods of Spackman *et al* (31) and Moore and Stein (27) with modifications by Niederwieser and Pataki (29), Blackburn (10) and Hirs (21). Amino acid analyses were performed on a Technicon Auto Analyzer (NC-2P) with a 25 cm column. An electronic integrator (Columbia Scientific Supergrator 2) was used to compute the absolute amounts of each amino acid.

Protein content was assayed by microkjeldahl according to Hiller *et al* (20), and the moisture content was determined using procedures described by Chibnall *et al* (13).

Table 15-3. Composition of the Artificial Diets, CMP-1 and CMP-2

Composition	Percent Composition CMP-1	Percent Composition CMP-2
Casein	40.00	40.00
Gelatin	10.00	10.00
Cod liver oil	10.00	10.00
Sucrose	10.00	10.00
Dextrin, white Technical	10.00	10.00
Cellulose[1]	4.26	7.16
Choline chloride (70%)	0.30	0.30
L-glutamic-HCl	1.20	–
L-ascorbic acid	0.30	0.30
L-glutamic acid	6.30	–
NaCl	0.50	0.50
Mineral mixture[2]	6.40	6.40
Vitamin mixture[3]	0.50	0.50
DL-Methionine	0.20	0.20
L-tryptophan	0.04	0.04
Amino Acid mixture[4]	–	4.60

[1] Solka floc, Brown Company, Berlin, N.H.

[2] Minerals in g/kg diet: $CaHPO_4H_2O$, 18.04; $CaCO_3$, 19.04; KH_2PO_4, 14.03; $NaHCO_3$, 8.82; $MnSO_4 \cdot H_2O$, 0.35; $FeSO_4 \cdot H_2O$, 0.50; $MgSO_4$,3.02; KIO_3, 0.01; $CuSO_4 \cdot H_2O$, 0.03; $ZnCO_3$, 0.15; $CsC1_2 \cdot 6H_2O$, 0.002; $NaMoO_4 \cdot H_2O$, 0.008; and Na_2SeO_3, 0.002.

[3] The vitamin mixture included 10,000 IU Vitamin A as retinyl palmitate; 4,000 IU Vitamin D as Cholecalciferol; 75 IU Vitamin E as dl-α-tocopheryl acetate; and the following amounts of vitamins in mg/kg diet: thiamin·HCL, 40.0; menadione dimethylpyrimidinol bisulfite (Vitamin K), 2.0; riboflavin, 30.0; D-calcium pantothenate, 150.0; niacin, 300.0; pyridoxine·HCL, 20.0; d-biotin, 0.5; folic acid, 15.0; Vitamin B_{12}, 0.3; Ethoxyquin (100%), 200.0; and myo-inositol, 500.0.

[4] Amino acids in g/kg diet: L-threonine, 7.0; L-valine, 5.0; L-cystine, 3.0; L-Isoleucine, 10.0; L-leucine, 8.0; lysine·HCL, 2.0; and L-arginine·HCL, 11.0.

Lipid Extraction and Analysis

Samples were collected, weighed, measured, lyophilized, and stored at -20°C under nitrogen. Several small fish or approximately one gram of each diet for each sample were rehydrated with 5 ml distilled water. Samples were extracted twice in a Sorvall Omni-mixer (60 ml capacity), according to the Bligh and Dyer (11) technique as modifed by Kates (23). Lipids were determined gravimetrically. The lipid material was saponified with 10 ml 0.5 N potassium hydroxide-methanol.

Fatty acids were methylated with 14 percent boron trifluoride-methanol (28). Fatty acid methyl esters (FAME) were injected into a single column Varian Aerograph 1200 gas-liquid chromatography unit operated isothermally at 180°C and equipped with a flame ionization detector. FAME were separated on a 15 percent diethylene glycol succinate (DEGS) column, on 100-120 mesh Chromosorb W-HP, 2.1 m long x 3.2 mm O.D., supplied with 75 ml/min flow of nitrogen as the carrier gas and a three percent ethylene glycol succinate polyester-Z (EGSP-Z) column (same dimensions as DEGS) on 100-120 mesh Gas Chromosorb Q support with 40 ml/min nitrogen. Identification and quantification of the FAME were made with an electronic integrator (Hewlett Packard 3380A) supplied with the relative retention times of authentic standards and literature values for published oils (2). Cod liver oil was used as a secondary standard (3) and heptadecanoic acid (17:0) was used as an internal standard (16). Unresolved chromatogram peaks were detected by comparing the profiles of the two individual column separations.

RESULTS AND DISCUSSION

Artemia Diet—Batch Culture

The total protein levels and amino acid profiles are given in Table 15-4 for the wild silversides, their eggs, and laboratory cultured fish of various ages. The cultured fish had been fed the live 3-day-old brine shrimp diet. The amino acid spectrum of the silversides was very similar to the spectrum of migrating Atlantic salmon (15).

The brine shrimp analysis was similar to the results of Gallagher and Brown (18) who also analyzed San Francisco Bay brine shrimp. These authors stated that methionine in the brine shrimp may be limiting compared to standard egg albumin levels. However, our results showed that the methionine levels in brine shrimp were very similar to the level found in the silverside eggs. The major differences between the 3-day-old brine shrimp and the silverside eggs were the lower levels of threonine, serine, proline, valine, and leucine, and the higher levels of arginine in the brine shrimp.

The silverside eggs contained higher levels of threonine, serine, proline, alanine, leucine, and tyrosine, and lower levels of glycine and methionine than were found in the wild fish. The amino acid profile of the 5-day-old silversides changed substantially from the profile of the eggs. Most of the changes resulted in a general decrease in amino acids from the egg to the larval stage.

Table 15-4. Amino Acid Profiles of Silversides, Expressed as Gram Amino Acid Per 100 Gram Protein

			Cultured Silversides				
Amino Acid	Wild Silversides	Eggs Unfert.	5-day	10-day	25-day	58-day	Brine Shrimp Diet
Aspartic Acid	9.3	8.7	10.2	9.9	9.9	10.2	8.9
Threonine	4.3	6.4	4.6	5.1	4.7	4.4	3.1
Serine	4.3	8.2	4.9	4.9	5.2	4.6	4.4
Glutamic Acid	14.1	13.7	15.2	14.4	15.2	13.8	11.6
Proline	3.9	7.5	4.2	4.3	4.5	4.8	3.1
Glycine	6.0	3.7	5.8	6.0	6.4	6.6	4.3
Alanine	6.0	7.7	4.7	4.5	4.9	6.2	6.0
Valine	5.3	6.8	5.5	5.6	5.8	5.6	5.1
Methionine	3.6	2.1	2.5	2.8	2.2	3.3	2.3
Isoleucine	4.6	5.9	4.8	5.1	4.4	4.0	4.9
Leucine	7.7	10.6	7.7	7.8	7.2	7.5	8.9
Tyrosine	3.5	5.1	3.9	4.0	3.9	3.2	4.2
Phenylalanine	4.2	4.7	4.7	4.6	4.6	4.3	4.5
Histidine	3.6	2.9	2.9	2.9	2.7	5.1	1.2
Lysine	8.9	8.3	8.3	8.7	8.3	8.9	9.2
Arginine	6.3	7.1	7.3	7.1	7.3	6.5	10.5
% Protein[1]	15	NA[2]	13	12	NA	14	NA
% Moisture	NA	84	79	81	81	79	NA

[1] wet weight basis

[2] NA = not available

Tryptophan was not determined by the procedure used in this study.

Table 15-5. Fatty Acid Composition of Unfertilized Eggs and 2- and 15-Day-Old Silversides Fed 3-Day-Old Brine Shrimp Nauplii

Fatty Acids	Unfertilized Eggs	Wild Silversides (20.5 mm)	2-Day-Old Sac fry (6.0 mm) Silversides	15-Day-Old Silversides (11.0 mm)
14:0	2.50[1]	1.34	1.14	0.69
14:1	0.27	0.14	0.06	0.30
15:0	0.77	0.55	0.36	0.33
15:1	0.15	0.14	0.02	0.19
16:0	18.67	22.55	22.67	16.29
16:1	7.40	5.83	4.48	10.06
17:1	1.28	0.14	0.13	1.49
18:0	5.99	9.44	9.74	7.34
18:1ω9	14.19	10.40	12.83	25.33
18:2ω6	1.58	1.23	0.68	2.24
18:3ω6	0.55	0.38	0.32	0.52
18:3ω3	1.47	0.88	0.44	2.49
18:4ω3	0.88	0.75	0.47	0.34
20:1ω9	1.36	0.71	0.20	0.49
20:4ω6	2.71	3.75	2.52	5.13
20:5ω3	8.09	7.37	4.90	7.84
22:5ω3	3.72	1.34	2.33	3.26
22:6ω3	27.15	35.53	36.05	14.96
% oil[2]	13.9	8.5	NA	NA
ω3/ω6 Ratio	8.5	8.6	12.6	3.7

[1] weight percent

[2] based on dry weight

From the fifth to the 58th day of culture, the amino acid profiles did not change markedly. The only changes which occurred were a decrease in glutamic acid and an increase in alanine and histidine. Compared to the 3-day-old brine shrimp diet, the 58-day-old fish differed only in the levels of histidine and arginine. Therefore, it seems that the dietary amino acids were absorbed and deposited as early as the fifth day of life.

Table 15-5 shows the fatty acids of unfertilized silverside eggs and 2 and 15-day-old fry fed on 3-day-old brine shrimp nauplii. The unfertilized eggs had a whole body lipid level of 13.9 percent and the fatty acids 20:5ω3 and 22:6ω3 comprised more than 35 percent of the total fatty acids. The ω3 acids exceeded the ω6 component by greater than eight times. It would appear that the high energy level coupled with the large ω3 polyunsaturated fatty acid (PUFA) component are indicative of their metabolic and physiological importance in the early life stages of silversides.

In the 2-day-old yolk sac fry the acids 16:0, 18:0 and 22:6ω3 were preferentially retained from the energy rich egg, while 16:1, 18:1ω9, 20:5ω3 and those acids which comprise individual contributions of less than 4 percent each showed reduced levels. The ω3/ω6 ratio of the 2-day-old yolk sack fry increased to 12.6, from the egg level of 8.5. A similar pattern of fatty acid retention and utilization was found by Hayes (19) and his associates in the total lipids of developing steelhead trout, *Salmo gairdneri*.

The brine shrimp diet was composed largely of 16:0, 16:1, 18:1ω9, and 20:5ω3 but contained no 22:6ω3 (Table 15-6). The analyses of silversides fed this diet (Tables 15-5 and 15-6) showed that the fatty acids 16:1 and 18:1ω9 increased from the 2-day-old yolk sac fry levels, while 16:0 and 22:6ω3 decreased. It is evident that the fish change their concentration of fatty acids to reflect the general composition of their diets. Other researchers have made the same correlation between the diet and tissue fatty acids of cultured fish (1, 9, 14, 24, 25).

In silversides cultured for 137 days (30) the level of 20:5ω3 and 22:6ω3 represented as little as three percent of the total fatty acid composition. The level of these two fatty acids in the wild fish represent an amount about ten times this level. Additionally, the wild fish had an oil content of only about eight percent, whereas 137-day-old cultured fish had a lipid level of 21.4 percent (30). Thus, brine shrimp fed fish did not closely resemble the lipid content of their natural counterparts. Since the ω3 acids have been shown to play a chief role in the metabolism of fish, it would seem that the amount of lipid storage may be related to a certain minimal amount of ω3 PUFA, namely 22:6ω3. A mechanism may exist which enhances the absorption and deposition of lipids to ensure a minimal 22:6ω3 tissue level. Therefore, the

Table 15-6. The Major Fatty Acids of the 3-Day-Old Brine Shrimp Diet and 25 and 58-Day-Old Silversides

FAME	3-Day-Old Brine Shrimp (Diet #2)	25-Day-Old Juvenile Silversides (13.05 mm long)	58-Day-Old Juvenile Silversides (22.24 mm long)
16:0	11.45	16.77	21.05
16:1	16.49	8.35	12.76
18:0	4.10	8.72	9.19
18:1ω9	34.34	27.44	36.87
18:2ω6	4.78	2.36	3.19
18:3ω3	4.67	2.85	2.26
20:1ω9	0.55	0.56	0.70
20:4ω6	3.13	5.99	3.79
20:5ω3	13.31	7.89	3.87
22:5ω3	–	2.23	1.73
22:6ω3	–	13.67	1.97
% oil	10.00	12.90	12.40
ω3/ω6 ratio	2.27	3.19	1.41

3-day-old brine shrimp diet may lead to critical nutritional problems if used in a long term study.

When compared to the wild fish (Table 15-5), the cultured fish have a far lower ω3 fatty acid level and much higher level of the ω6 acids. The ω3/ω6 ratio of the wild fish lipid was 8.0, more than two times the cultured fish levels. The wild fish fatty acid profile was similar to the egg and 2-day-old yolk sac fry values, as would be expected. In the wild fish the fatty acids 20:5ω3 and 22:6ω3 represented about 40 percent of the total fatty acid composition. Preferably, the cultured fish should resemble the wild juvenile fish in our experiments.

Artificial Diets

Since the amino acid profiles of the Atlantic salmon and wild silversides were comparable, a commercial salmon diet was tried in the preliminary evaluation of the artificial diets. Compared to the brine shrimp fed fish, growth and survival in the test diet fed group was very poor. Fish on the salmon type diet exhibited some scoliosis. Two factors which could have contributed to this problem were the leaching of dietary components from the artificial diet when it became water soaked, or perhaps an inadequate lipid composition (soybean oil). In reference to the latter point, the fatty acid composition of the artificial diet is shown in Table 15-7 along with the spectrum for soybean oil and that of silversides cultured on the salmon type diet. The soybean oil diet and the oil resemble each other to some degree, since 16:0, 18:1ω9, and 18:2ω6 are the major fatty acids of both analyses. Likewise, silversides fed this artificial diet closely resemble the lipid make-up of the diet they were fed. However, it is evident that fish fed the artificial diet were not similar in fatty acid composition to the wild fish, and thus this diet had not accomplished the major goal of providing a laboratory cultured fish of similar biochemical composition to the wild fish (Table 15-5).

Based on the biochemical analyses of the diets and cultured fish, and the results of poor growth and survival, the salmon diet was modified by the addition of a marine oil (cod liver oil). The soybean oil based diet was again fed to verify past results. In addition to these two diets, the live brine shrimp diet was used along with: a freeze-dried form of brine shrimp, a wild plankton diet, Tetra Marin—a commercial aquarium food, and two semi-purified diets with various amino acid compositions (Table 15-3). The cultured fish were again analyzed for protein and lipid composition.

The growth and survival results of fish fed these experimental diets are presented in Table 15-8. The live brine shrimp diet gave the best survival (97

Table 15-7. The Major Fatty Acids of Soybean Oil, an Artificial Diet (#7) with a Soybean Oil Base, and Silversides Cultured on the Diet

FAME	Artificial Diet With Soybean Oil	Soybean Oil	Silversides Fed the Artificial Diet (13.97 mm long)
16:0	13.05	12.52	17.43
16:1	2.43	–	3.74
18:0	2.45	4.69	7.41
18:1ω9	36.12	19.25	36.37
18:2ω6	29.81	54.90	21.22
18:3ω3	1.88	7.83	0.51
20:1ω9	2.94	–	2.95
20:5ω3	1.62	–	–
22:6ω3	2.08	–	4.76
ω3/ω6	0.19	0.14	0.25

Table 15-8. Percent Survival and Weight Gain for Silversides Cultured on Various Experimental Diets (Modified from Beck and Poston (7))[1,2]

Experimental Diet	Survival %	Weight Gain mg	 %
1. Wild plankton	54.0	4.3	48.3
2. Brine shrimp-live	97.0	36.6	411.2
3. Starved	0.0	–	–
4. Brine shrimp-dried	66.0	4.5	50.6
5. Tetra Marin	95.0	4.4	49.4
6. Artificial, HPM-1	51.0	0.6	6.7
7. Artificial, CHP-1	28.0	1.2	13.5
8. Artificial, MP-1	65.0	-0.7	-7.9
9. Artificial, MP-2	61.0	-0.5	-5.6

[1] Diet #1 data only one replicate, diets 2, 4-9 are averages of two replicates.

[2] Fish were 23 days old at onset of the study and were cultured for 23 days.

percent) and the best weight gain (411 percent). Normal growth of the wild fish has been estimated at 12 mm per month during the growth period (6). None of the other diets gave an appreciable weight gain; in fact some groups actually lost weight. With the exception of Tetra Marin, all the artificial diets produced a relatively poor survival rate.

Table 15-9 shows the fatty acid composition of the natural and artificial diets. The effect of the dietary fatty acids on the cultured fish lipids is presented in Table 15-10. The diet profiles and the respective cultured fish profiles were quite similar. The cultured fish fed the cod liver oil based diets (Diets 6, 8, 9) and those fed Diet 5 contained a much higher level of $22{:}6\omega3$ than the brine shrimp fed fish (Table 15-6 and 15-10) or the soybean oil fed fish (Table 15-7 and 15-10). These fish more closely resembled their wild fish counterparts. A lipid modification of the salmon diet had therefore effected a biochemical change in the fish to a more "wild like" laboratory fish. The replacement of the soybean oil in Diet 7 by cod liver oil (Diet 6) doubled the survival level, however growth was only one-half as great. It is very difficult to draw a direct correlation between the dietary lipid composition of the various diets and survival, since the experimental design of this study was quite unlike the classical nutrition experiments.

The comparison of the amino acid profiles of fish fed the natural and artificial diets indicated little variation between the treatment groups (Table

Table 15-9. Percentage Composition of Fatty Acids from Total Lipids of the Various Experimental Diets

Fatty Acid	Experimental diets #1	#2	#4	#5	#6	#7	#8	#9
14:0	7.42	1.16	1.33	1.39	4.50	1.90	4.39	4.72
14:1	1.64	1.10	1.14	0.14	0.40	0.16	0.51	0.46
15:0	0.87	0.53	0.57	0.18	0.31	0.15	0.33	0.30
15:1	0.15	0.38	0.36	0.06	0.09	0.02	0.13	0.12
16:0	26.30	11.45	12.27	16.83	13.17	13.00	10.84	11.59
16:1	10.50	16.49	17.05	4.74	9.03	2.45	11.21	11.48
16:4	0.45	–	–	0.06	–	0.08	0.40	0.19
17:1	0.24	2.72	2.55	–	0.18	0.06	0.27	0.20
18:0	5.24	4.10	2.16	3.64	0.66	2.45	2.01	2.02
18:1ω9	9.48	34.46	36.43	22.40	24.36	35.56	25.05	26.73
18:2ω6	1.93	4.78	2.98	31.38	7.64	29.52	3.66	2.83
18:3ω6	1.13	0.75	0.81	–	–	–	–	–
18:3ω3	2.70	4.67	4.49	5.31	1.05	1.90	1.04	0.81
18:4ω3	3.46	0.73	0.75	0.25	1.99	0.70	3.89	2.12
20:0	0.13	–	0.03	0.18	–	–	–	–
20:1ω9	0.36	0.55	0.62	3.43	9.40	3.49	9.77	10.28
20:2ω6	0.40	–	0.04	0.08	0.19	–	0.27	0.29
20:4ω6	1.22	3.13	2.72	0.36	0.33	0.11	0.26	0.30
20:5ω3	12.58	13.31	12.20	3.46	8.36	1.48	9.94	9.45
20:0	–	–	–	–	0.36	–	0.39	0.43
22:1	–	–	–	1.99	6.95	4.41	5.03	5.01
22:5ω6	–	–	–	–	0.04	–	–	–
22:5ω3	0.03	–	–	0.21	0.62	0.06	0.81	0.81
22:6ω3	13.44	–	–	3.72	9.69	1.99	10.60	10.09
ω3/ω6 ratio	6.88	2.16	2.66	0.41	2.65	0.21	6.27	6.81

Table 15-10. Percent Composition of the Fatty Acids from Total Lipids of Silversides Cultured for 23 Days on the Various Experimental Diets.

Fatty Acid	23-Day Old	Experimental Diets 1	2	4	5	6	7	8	9	Wild Fish
14:0	0.53	1.26	1.39	0.90	1.08	2.27	1.46	2.53	3.51	1.34
14:1	0.29	–	0.32	0.44	–	–	0.06	0.17	–	0.14
15:0	0.32	0.56	0.51	0.66	0.52	0.45	0.35	0.45	0.83	0.88
15:1	0.09	0.18	0.14	0.31	0.33	0.15	0.18	0.27	0.48	0.14
16:0	16.77	21.43	15.66	16.91	19.28	17.94	17.95	19.56	20.68	22.55
16:1	8.35	4.13	13.09	8.51	3.14	5.23	2.92	6.36	4.64	5.83
17:1	0.90	–	0.63	1.33	1.88	0.98	0.11	0.99	–	0.14
18:0	8.72	13.31	6.32	6.09	13.58	7.40	7.99	9.16	9.53	9.44
18:1ω9	27.44	12.52	33.83	27.22	15.41	24.27	34.20	24.72	24.60	10.40
18:2ω6	2.36	1.30	3.45	2.85	13.54	3.06	18.39	2.78	1.82	1.23
18:3ω6	0.42	1.95	1.14	1.45	1.71	1.31	2.45	0.58	0.58	0.38
18:3ω3	2.85	0.56	3.05	1.92	1.48	0.48	0.52	0.18	0.80	0.88
18:4ω3	0.26	0.30	0.44	0.76	0.46	0.42	0.21	0.36	0.36	0.75
20:0	0.20	0.38	0.03	0.21	1.34	–	–	0.18	0.15	–
20:1ω9	0.33	1.14	0.79	1.35	1.74	7.81	2.78	6.10	5.58	0.71
20:2ω6	–	0.46	–	0.06	0.29	0.68	0.08	0.05	0.15	0.09
20:4ω6	5.99	3.32	3.93	5.37	3.49	2.66	1.48	2.97	2.54	3.75
20:5ω3	7.89	5.14	4.60	6.89	1.76	3.00	0.44	2.42	2.22	7.37
22:1	–	0.14	–	0.37	0.51	3.91	1.27	2.28	2.44	0.20
22:4ω6	–	0.27	–	–	0.29	–	–	–	0.03	0.24
22:5ω6	–	–	0.03	–	–	–	–	–	–	–
22:5ω3	2.23	2.60	4.70	2.63	1.30	0.55	–	0.65	1.47	1.35
22:6ω3	13.67	29.03	5.83	13.57	16.62	17.47	6.95	17.24	17.99	35.53
% oil	12.88	11.27	19.90	10.79	11.72	12.82	NA	15.45	12.44	NA
ω3/ω6 ratio	3.07	5.15	2.18	2.65	1.12	2.84	0.36	3.27	4.46	8.06

15-11). Although histidine and methionine were present in greater amounts in Diets 6 and 9 than in the other treatment groups, no relationship is obvious between these amino acid levels and growth and survival. The higher amounts of leucine in the brine shrimp fed fish could indicate a role of this amino acid in their greater growth and survival. However, this was not evident in the freeze-dried brine shrimp fed group.

No substantial differences were found in growth and survival of fish fed the nonsupplemented amino acid diet (Diet 8) versus the supplemented diet (Diet 9). Therefore, it is difficult to say the quantity of dietary amino acids influence the metabolism and utilization of the various diets. Bioavailability studies will be necessary to ascertain the degree of incorporation of the supplemented amino acids.

CONCLUSIONS

1. The best growth and survival of juvenile silversides was obtained on a live 3-day-old brine shrimp nauplii diet. Substantially lower growth and survival was obtained on the artificial diets.

2. Freeze-dried brine shrimp provided less growth than a live brine shrimp diet. Live brine shrimp must contain some component which is removed or altered upon freeze drying.

3. It is difficult to say whether protein (amino acids) in the diets was a factor in the differences in growth and survival of cultured fish fed the artificial diets and the brine shrimp fed fish. The brine shrimp fed fish did, however, contain higher levels of leucine than all other cultured groups. Bioavailability studies will be necessary to ascertain the degree of assimilation and incorporation of dietary amino acids.

4. Whole body lipid fatty acid composition of cultured fish changed to reflect the composition of their diets. Fish fed the brine shrimp diet had higher fat levels and lower polyunsaturated fatty acid levels than wild fish. Cultured fish appear to be storing large amounts of lipids in order to obtain a threshold level of the polyunsaturated fatty acids.

5. Fish fed a cod liver oil based diet more closely resembled their wild counterparts. However, growth and survival were poor compared to 3-day-old brine shrimp fed fish.

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Table 15-11. Amino Acid Profiles of Silversides Fed the Various Artificial and Natural Diets Expressed as Gram Amino Acid Per 100 Gram Protein

	Experimental Diets						
Amino Acid	1	2	4	5	6	8	9
Aspartic Acid	10.1	9.9	10.5	10.2	10.2	9.7	10.0
Threonine	4.5	4.0	4.9	4.4	4.4	5.0	4.3
Serine	4.5	4.2	5.0	4.7	4.7	5.0	5.4
Glutamic Acid	15.4	16.3	14.8	15.4	14.3	15.2	14.1
Proline	3.9	3.7	4.0	4.6	4.6	4.5	4.7
Glycine	6.7	6.8	7.0	6.6	7.2	6.0	6.4
Alanine	6.4	6.7	6.4	7.4	5.2	5.6	6.2
Valine	5.2	4.5	5.0	5.1	5.4	5.6	5.7
Methionine	1.5	2.4	1.5	2.5	1.9	2.3	3.2
Isoleucine	5.1	4.6	4.8	4.8	4.5	4.2	4.5
Leucine	7.9	11.4	7.8	7.4	7.7	8.1	7.8
Tyrosine	2.3	3.2	2.4	3.3	3.7	3.1	3.5
Phenylalanine	4.5	4.2	4.6	4.4	4.8	4.8	4.9
Histidine	3.3	2.7	3.5	3.4	4.7	3.8	3.3
Lysine	8.5	9.2	9.1	9.0	8.6	7.9	8.1
Arginine	7.3	6.2	7.4	7.3	6.9	7.0	7.1
Ammonia	19.6	16.2	17.8	16.1	17.4	18.4	16.9
% Protein[1]	19	17	17	16	13	15	14
% Moisture	76	75	NA[2]	77	NA	77	NA

[1] Net weight basis

[2] NA = not available

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THE COMBINED EFFECT OF TEMPERATURE AND DELAYED INITIAL FEEDING ON THE SURVIVAL AND GROWTH OF LARVAL STRIPED BASS *Morone saxatilis* (WALBAUM)

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ABSTRACT

Rearing temperature and the time of first feeding interact to determine the degree of survival and rate of growth in larval striped bass. Between 15 and 27°C, temperature affects the rate of growth and development in fed groups, and the time to death by starvation in unfed lots. Delayed first feeding retards structural development. The 'point-of-no-return' in striped bass is very near the stage of complete mortality due to starvation. Unfed groups survived up to 22 days after hatching at 24°C and 32 days at 15°C. Larvae fed late into starvation survived and continued to grow at a rate somewhat higher than that observed in earlier fed groups at all temperatures. Larvae which has survived delayed development were indistinguishable on the basis of external morphology from much younger individuals reared under more favorable conditions. The effects of nutritional and thermally induced developmental retardation are discussed in terms of how they may affect larval growth and mortality rate estimates used in assessing the effects of estuarine power plants.

INTRODUCTION

Many estuarine and marine fish species, including the striped bass, *Morone saxatilis* (Walbaum), produce large numbers of relatively small pelagic eggs at spawning. These smaller eggs contain fewer yolk reserves. After a relatively short incubation period, they hatch into prolarvae that are, in general, at a more rudimentary stage of structural development than those of species producing fewer but larger eggs (3, 17). Development continues while the young larvae drift in the water column, absorbing their yolk and developing the mouth parts and swimming ability to capture food and avoid predators. Although the methods used to determine the extent of first year mortality in natural populations are at best imprecise (25), it is clear that among high fecundity species, losses early in life are extremely high, with the highest mortality rates among the early larval stages.

As early as the end of the last century, Fabre-Domergue and Bietrix (9) encountered heavy mortality among laboratory reared marine fish larvae which had exhausted their yolk reserves. Hjort (12) concluded, based on his studies of year to year fluctuations in Norwegian cod and herring abundance, that year-class strength was probably determined early in the larval development of these species. The term "critical phase" or "critical period" has been used, in a general sense, to refer to that span of time in the early development of the individuals comprising a particular year-class during which the ultimate number of recruits is determined (11). In a narrower usage "critical period" may be used to refer to that point in development of the larval fish at which all sources of endogenous (yolk) nutrition have been consumed, and active feeding must commence if death by starvation is to be avoided. Hjort (12) proposed death following yolk exhaustion as only one of several possible mechanisms by which events early in development might affect the subsequent size of a given year-class. In 1956, Marr (23) reviewed the available evidence in support of the existence of a "critical period". He concluded that there was little evidence to suggest that mass starvation occurred in the sea among larvae that had recently absorbed their yolk, or that survival curves for natural populations revealed any noticeable inflection at the point of yolk absorption. 18 years later, May (25) noted that little new data has been gathered since Marr's review that could contribute meaningfully toward the resolution of the problem of whether or not a "critical period" at yolk absorption exists as a widespread phenomenon among fish species. He suggested that while among high fecundity species, year-class strength is certainly determined during early development as Hjort maintained, the physiological mechanisms that have evolved to meet environmental challenges that confront the developing larva must be addressed on a species by species basis.

The prolarva, from the time it is hatched until it captures its first meal, is reliant on its yolk reserves to provide the structural materials for continued ontogenetic development, as well as to provide energy to fuel its maintenance, activity, and growth needs. Unless sufficient satisfactory food is taken after the exhaustion of yolk reserves, structural tissue already laid down is metabolized to support the continued costs of swimming in search of prey, until the larvae is so debilitated by the effects of starvation that it is unable to capture and utilize suitable prey when it does become available. Blaxter and Hempel (4) termed this ecological death-point the "point-of-no-return" (PNR). Starved larvae may live after the PNR has been reached but with no likelihood of ultimate survival. The time span between the development of the ability to feed and PNR determines how important the period of transition to exogenous feeding will be to the survival of larvae of a particular species.

The rate of growth and development of larval fish is very much temperature dependent. Among the studies in which the relationship between rearing temperature and larval growth rate have been demonstrated are those of Kramer and Zweifel (16), Houde (14), and Shelbourne *et al* (33). In all these studies, larval growth rate increased with increasing temperature, except where survival limits were approached.

Weight specific metabolic rate (oxygen consumption) also increased with increasing temperature among fish larvae of the same weight in studies such as those of Holiday *et al* (13) and Laurence (20).

The striped bass, *Morone saxatilis* (Walbaum), is a commercially important anadromous teleost native to the Atlantic coast of North America. The natural range of the striped bass extends along the Atlantic coast of North America from the St. Lawrence River to Louisiana, with its center of abundance between Cape Cod and Cape Hatteras (26). There have been many introduced populations ranging from the extremely successful Pacific coast estuarine population, introduced in the 1880's, to the many landlocked populations which have been established in natural and man-made freshwater impoundments in the southeastern states.

Sexually mature striped bass enter and ascend rivers to the spawning grounds within the period between March and July. Peak spawning generally occurs at a water temperature on the spawning grounds of 15 to 18°C. Spawning sites are typically well into the freshwater portion of the estuary, although often within the range of tidal influence (35). Each ripe female may produce from one to three million eggs. Newly shed eggs are 1.28 to 1.38 mm in diameter. Upon water hardening they swell to a diameter of approximately 3.0 mm. Newly hatched larvae average 3-4 mm in length, and have a large yolk sac and oil globule (22). For several days after hatching, young prolarvae spend much of their time in a vertical, head-up position drifting in the current. Larvae develop functional mouth parts and are capable of feeding within 2-10 days after hatching at normally encountered river temperatures. Yolk is generally completely absorbed by the time the larva reaches 6 mm in length. Metamorphosis into essentially adult form occurs by the time the larvae are approximately 17 mm in length, generally 2 to 3 weeks after hatching. Feeding larvae are capable of consuming relatively large organisms as their first food. Planktonic crustacea and their developmental stages predominate in their diets through most of their first year (27, 10).

The striped bass is a well-studied species and there is a voluminous literature relating to its biology (30). Relatively little attention, however, has been paid to the ecology of early life stages, the period during the life of the animal which is most important in the determination of year-class strength. Mansueti (22) presented descriptions of the eggs and larvae from collections from the Roanoke and Patuxent Rivers, and provided observations on the feeding and early growth of larvae in captivity. Doroshev (8) reviewed aspects of egg and larval development and added anecdotal observations on metabolic rate and graying rates of larvae. Bayless (2) provided a manual of culture methods as practiced in South Carolina hatcheries, but did not give many details of larval requirements beyond the yolk sac stage. Short term lethal temperature levels for eggs and larvae were presented by Albrecht (1), Davies (7), Shannon and Smith (32), Shannon (31), and Morgan and Rasin (28). Observations of prey selectivity among late larvae were reported by Meshaw (27) and Gomez (10). Daniel (6) presented data on the effect of food density on larval survival.

In many spawning rivers on the Atlantic coast, major conflicts have arisen over the effect of power plant operations on striped bass recruitment, as a result of the entrainment of eggs and larvae in cooling water intakes, and later the impingement of juveniles on intake screens. Entrainment losses are highest among striped bass under approximately 3 cm in length. The duration of the period of major entrainment losses is a direct function of the time required for young bass to develop from semiplanktonic eggs to early juveniles large enough to escape intake currents. An assessment of plant impact must take into account the duration of entrainable life stages. To date, only crude estimates have been used in plant impact models involving striped bass (e.g., 21, 36).

The purpose of the present study was to determine in what way temperature and an initial delay in the onset of active feeding work together to affect the rate of survival and growth of striped bass larvae. Temperature is a controlling factor which may be expected to have a profound effect on the metabolic demands of the developing larva. The availability of food determines the extent to which these demands can be met. Temperature and delayed first feeding may be expected to interact in a manner which would largely determine the life span and early growth trajectory of the developing larva. By observing how water temperature and feeding level affect growth, better predictions of stage duration, hence vulnerability to power plant entrainment, may be made.

MATERIALS AND METHODS

Source of Study Material

Eggs from Maryland used in the 1976 experimental series were netted from the Nanticoke River during the spawning season, using a 1 x 2 meter, 947 micron mesh neuston net. Eggs were also obtained from a striped bass hatchery run by the state of South Carolina at Moncks Corner, South Carolina. Eggs were air-shipped to the University of Rhode Island, where all experiments reported here were performed.

Experimental Procedures

For the duration of the relatively short incubation period, eggs were maintained in static 208 liter polyethylene drums filled with dechlorinated tap water. Best hatching success was observed when bacteria were controlled using an antibiotic. The antibiotic dosage used was 50,000 I.U./liter penicillin G plus 50 mg/liter streptomycin sulfate. A strong air stream maintained the eggs in suspension and maintained an adequate dissolved oxygen level. Dead eggs floated to the surface and were removed as they were discovered. One-half of the volume of the tanks was replaced daily. Water temperature was maintained at laboratory room temperature, 14-16°C, during incubation.

The experimental containers used in growth experiments consisted of four liter glass beakers. Prolarvae were stocked into these containers usually within 24 hours of the time they were hatched. Larvae stocked at yolk absorption were held in their incubation containers until visible vestiges of yolk had disappeared. At stocking, all were of the same chronological age and had been exposed to the same conditions prior to the beginning of the experiment. No antibiotic was used in larval growth or survival experiments. The water used in all experiments was raised to 5°/oo salinity by mixing dechlorinated tap water with seawater (32°/oo, which had been passed through a cartridge filter rated to retain particles larger than 5 microns). Water in each container was changed every two days.

Constant temperatures of 15, 18, 21, 24, and in some cases 27°C, were maintained in the test containers by keeping them immersed in temperature controlled water baths. Bath temperatures were controlled using Haake (model E-52) 1000 watt heater-thermoregulators operating against a cooling coil in each bath. Temperature excursions of no more than 0.25°C were normally encountered. The temperatures used span the range that might be encountered by developing larvae in nature. Bath temperatures were monitored on a strip-chart recorder and measured manually at least twice a day during the course of experiments.

In initial experiments, dissolved oxygen, pH, ammonia, and salinity measurements were made regularly. Dissolved oxygen was determined using the Y.S.I. D.O. probe, supplemented periodically with determinations using the azide-modification of the Winkler titration. The pH was measured using an Orion pH electrode. Ammonia was determined using a micro-modification of the indophenol technique of Solorzano (34). Salinity measurements were made using an American Optical salinity refractometer. With frequent water changes, most of the water quality parameters changed insignificantly during the course of each experiment. In later experiments where the number of individual treatments became unmanageable, regular monitoring of all variables except temperature was discontinued, and a stringently maintained schedule of water changes observed.

Feeding larvae were supplied with newly hatched *Artemia* nauplii at least twice a day in quantities sufficient to permit a portion to remain until the next feeding. *Artemia* nauplii proved to be a satisfactory diet for striped bass through the early juvenile stage.

Larval growth was measured in terms of dry weight. Prior to weighing, larvae were lifted on a No. 6 sable brush, dipped in distilled water to remove any adherent salt or debris, blotted on filter paper and placed on a tared weighing pan. Pans were cut out of aluminum foil with a paper punch and ashed 4 hours at 500°C before use. Dry weight determinations were made after the specimen on its tared pan had been dried to a constant weight in a heated vacuum desiccator at 80°C over silica-gel. On larvae up to metamorphosis, weights were determined using a Cahn "Gram" electrobalance. Weights were read to the nearest microgram.

Design of Experiments

Temperature-delayed feeding-survival experiments were performed during the springs of 1976 and 1977. A total of three experiments were performed, each identical in its design. In each, five containers were placed in each of four constant temperature water baths. Each container was stocked with 100 prolarvae at the temperature at which they had been held prior to the beginning of the experiment. Stocked containers were assigned to particular temperature treatments by lot. The acclimation period from holding temperature to the experimental temperature treatment was at most one hour.

In each temperature treatment, the larvae in one container were offered a diet of newly hatched live *Artemia* nauplii at the beginning of the experiment. Food was withheld from another container throughout the observation period. The time of first feeding for larvae in each of the remaining three containers of starved larvae in each temperature treatment was determined on the basis of observations of the apparent state of health of individuals in each population. Each container was checked for mortality several times each day throughout each experiment, and all dead larvae removed.

Similar stocking and treatment procedures were used in temperature-delayed feeding-growth experiments. Food was withheld from one group at each temperature, and one group at each temperature was given food at the beginning of the experimental period. Initial dry weight measurements were made on a sample of 20 larvae at the beginning of the experiment. At the time each group was given its first food, a sample of 10 larvae from the unfed lot was weighed. At the end of the observation period all of the larvae in each treatment were measured and weighed. In cases where an intermediate growth observation was made between the time of first feeding, and before the end of the experiment, a sample of 10 larvae was used to establish growth of the population to this point.

RESULTS

Figure 16-1 shows the effect of delayed initial feeding on groups of larvae maintained at four temperatures. The survival time of the unfed control decreased with increasing temperature. The time to 50 percent mortality for unfed groups was 19, 21, 25 and 27 days after hatching among groups maintained at 24, 21, 18 and 15°C, respectively. Survival among early fed groups was generally highest in each temperature treatment. Among groups in which food was provided for the first time after up to 50 percent of the population had died, a portion of those remaining alive survived through the end of the observation period. A "point-of-no-return" beyond which survival could not occur even when food was provided, was nowhere in evidence in these experiments. In each temperature treatment the longer food was withheld, the greater the total mortality each group suffered. In cases where some additional mortality was observed after food was presented, there was generally evidence that the dead larvae had captured at least some food before expiring.

Figure 16-2 shows the result of an experiment in which initial feeding was progressively delayed in a series of experimental groups of larvae held at five test temperatures. Changes in dry weight were used here to measure the rate of growth or shrinkage in fed and unfed groups at each temperature. Among starved lots, longevity increased and the rate of weight loss decreased at lower temperatures. Growth in dry weight increased rapidly once food was provided. In general, the effect of delayed initial feeding was to defer the attainment of a temperature-specific rate of growth of which larvae fed to satiation were capable.

Larvae receiving their first food at day six after hatching at 15°C, had scarcely recovered their initial weight at hatching by the end of the 25-day observation period. Other groups which received their first food at day six after

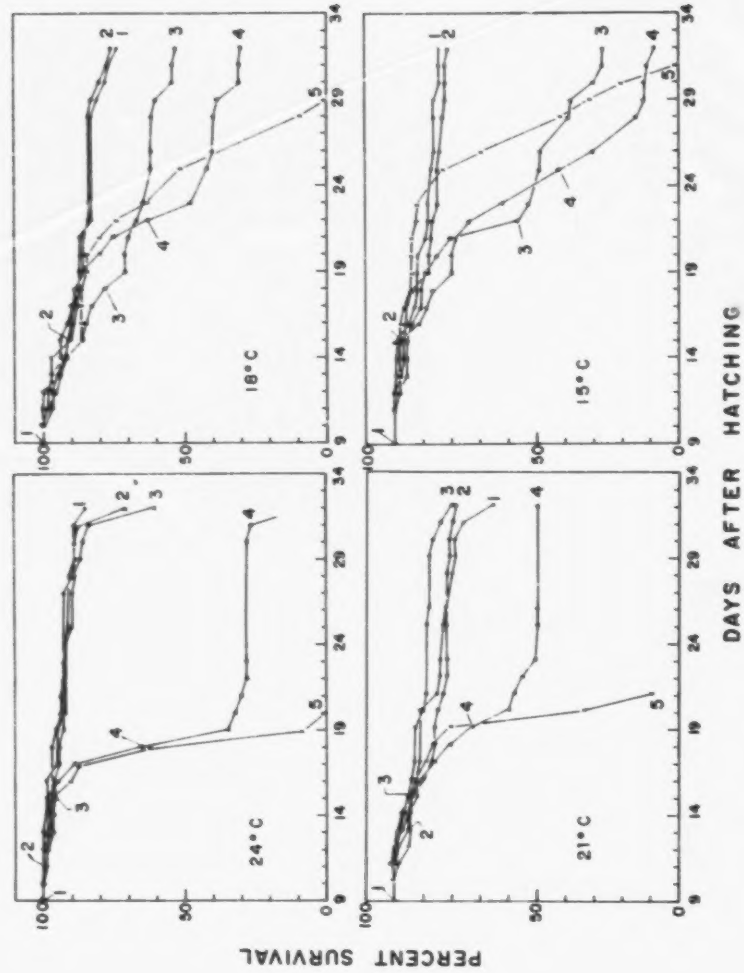

Figure 16-1. The Effect of Delayed Feeding on the Survival of Striped Bass Stocked at Yolk Absorption at 24, 21, 18 and 15°C.

NOTE: Initial population 100 larvae. Numbered arrows indicate the order and time of first feeding. Group 5 was unfed.

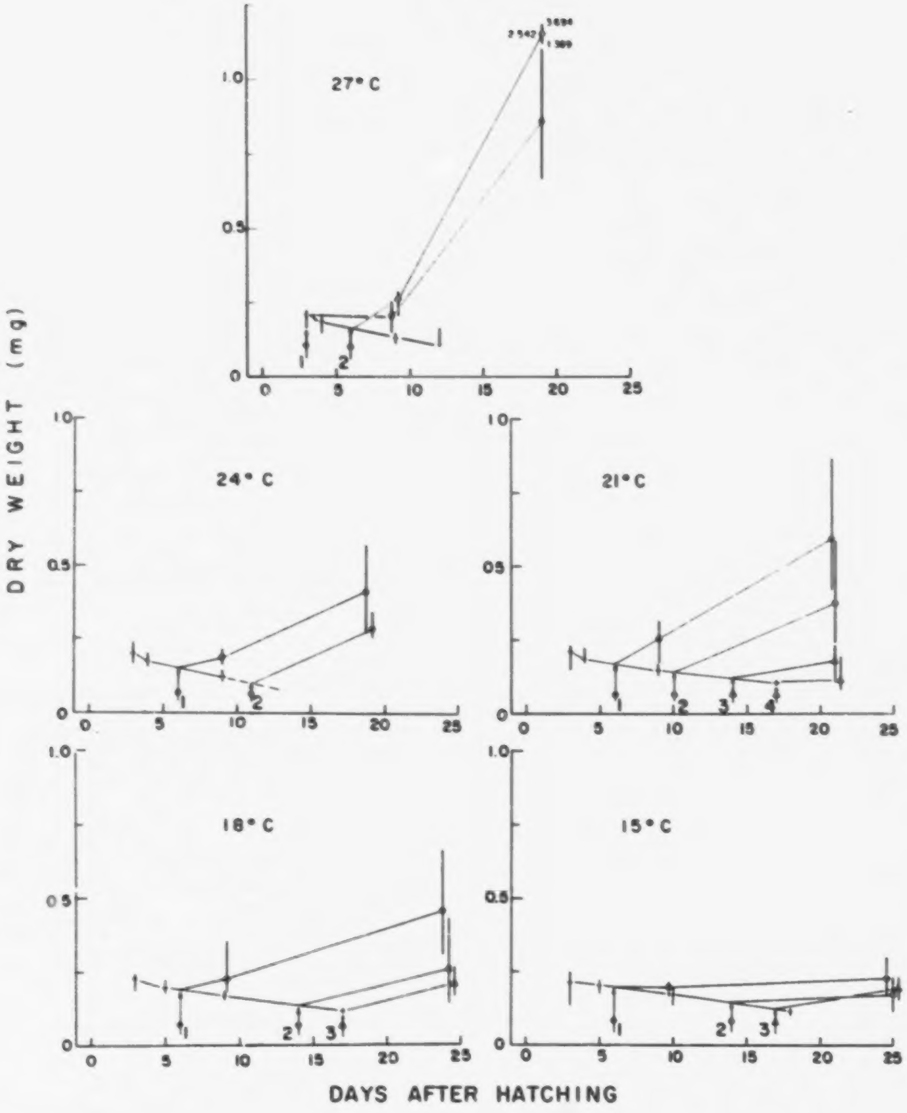

Figure 16-2. The Effect of Temperature and Delayed Feeding on the Growth in Dry Weight of Striped Bass Larvae Stocked at Hatching at 27, 24, 21, 18 and 15°C.

NOTE: Each sample contains 10 individuals. Numbered arrows indicate time of first feeding for each population. Symbols identify groups which received their first food at the same time. The location of symbols denotes sample means. Vertical bars indicate range of weights in each sample.

hatching, attained a mean dry weight of 0.5 mg at 25, 19, 18 and 12 days after hatching in temperature treatments of 18, 21, 24 and 27°C, respectively.

There was a tendency for later fed groups at each temperature to grow at a greater rate than those receiving their first food earlier in the experiment. Table 16-1 shows the instantaneous growth coefficients calculated for delayed feeding groups at each temperature, using the same data that were presented graphically in Figure 16-2. In each case, the growth rates of the earliest fed groups were lower than those of groups which had been starved before receiving their first food. This growth compensation seldom permitted later fed groups to overtake those fed earlier, but did serve to partially offset the growth setback that resulted from later initial feeding.

An additional effect of delayed initial feeding was a retardation of structural development which was observed at all temperatures. Figure 16-3 shows an example of the degree of developmeantal retardation which may occur among larvae of the same chronological age as a result of a delay in the timing of initial feeding. Among developing larvae, each developmental event appeared to coincide with the attainment of a particular larval length or dry weight. As a result, factors like temperature and nutritional state have a marked effect on the degree of structural development larvae of a particular age may achieve.

DISCUSSION

Among the fish species that have been investigated in the past, there appear to be several alternative patterns of survival following the delayed initial feeding of larvae which have consumed most or all of their yolk reserves. The northern anchovy, *Engraulis mordax* (Girard) (19) and the herring, *Clupea harengus* (4), both may survive food deprivation to a point beyond which continued survival is possible but ultimate recovery is not. The grunion, *Leuresthes tenuis* (Ayres) (24), on the other hand, can recover from food deprivation nearly up to the point of death through starvation. Observations reported here using striped bass and those of May (24) using grunion, are very similar. For neither species are the concepts of a "point-of-no-return" or of a "critical period" at yolk absorption appropriate. In the striped bass, as in the grunion, protracted food deprivation results in a suspension of further structural development, and a gradual reduction in dry weight during starvation as the costs of continued maintenance are met at the expense of body tissues.

The experiments of May (24) was performed at one temperature. In this study a range of temperatures was used. Temperature has been repeatedly shown to have a controlling influence on the rate of growth of fish larvae maintained on unlimited rations (e.g., 14, 16, 20). Similarly, temperature affects the rate of weight loss during starvation (18). Data presented in Figure

Table 16-1. Instantaneous Growth Coefficients for Delayed Feeding Groups at Five Constant Temperatures.

Temperature (°C)	Initial Dry Wt. (mg)*	Final Dry Wt. (mg)	Days Since Hatching: Day of First Feeding	Days Since Hatching: Day Measured	Instantaneous Growth Coefficient**
27	0.211	0.863	3	19	8.803
	0.157	2.542	6	19	21.419
24	0.155	0.413	6	19	7.538
	0.100	0.289	11	19	13.266
21	0.170	0.593	6	21	8.330
	0.140	0.376	10	21	8.981
	0.120	0.180	14	21	5.793
	0.102	0.111	17	21	2.114
18	0.190	0.451	6	24	4.802
	0.135	0.257	14	24	6.438
	0.116	0.205	17	24	8.135
15	0.198	0.231	6	25	0.811
	0.145	0.174	14	25	1.657
	0.125	0.195	17	25	5.558

*Determined by interpolation when the actual weight was not available (See Figure 16-2).

**Instantaneous growth coefficient (29) = $\frac{\log_e wt_2 - \log_e wt_1}{t_2 - t_1}$

where wt_1 and wt_2 are dry weight at times t_1 and t_2, respectively.

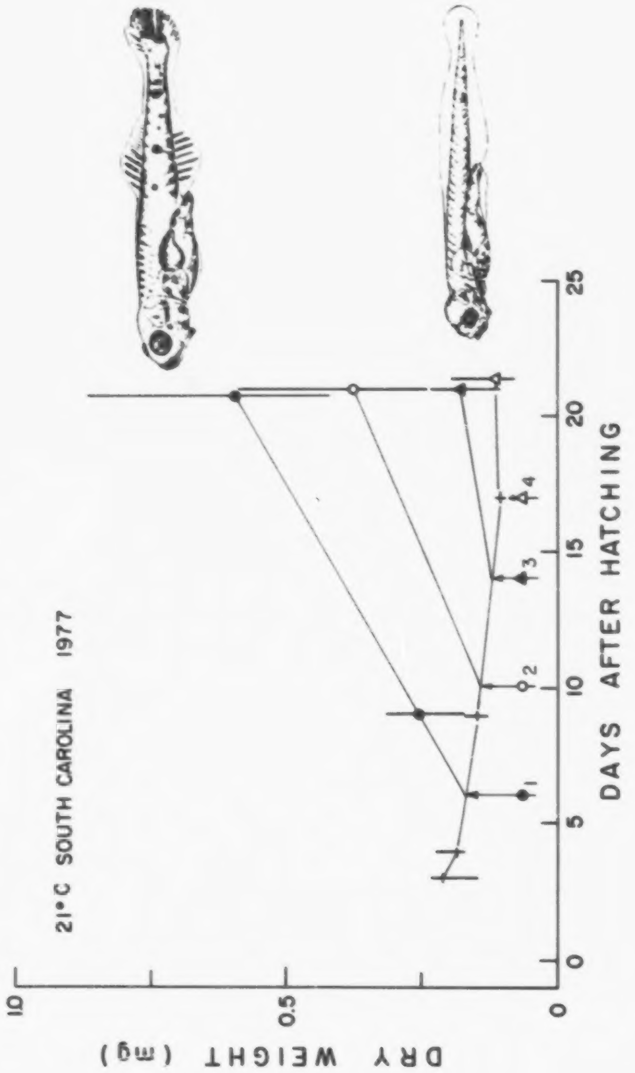

Figure 16-3. The Effect of Temperature and Delayed Feeding on the Growth in Dry Weight of Striped Bass Larvae Stocked at Hatching at 21°C.

NOTE: Each sample contains 10 individuals. Numbered arrows indicate time of first feeding for each population. Symbols identify groups which received their first food at the same time. The location of symbols denotes sample means. Vertical bars indicate range of weights in each sample. Development at twenty-one days after hatching is shown for larvae receiving their first food on days six and fourteen.

16-2 of this study indicate that at lower temperatures the differences in size between early-fed and starved larvae was not great even three weeks after hatching. At higher temperatures both the rate of weight loss in starved populations, and the rate of weight gain in early fed populations increased markedly, with the major effect being seen on the rate of individuals in groups which were fed an unrestricted ration shortly after yolk absorption.

There are vast differences between the conditions that exist in the laboratory and those in the natural habitat of the striped bass. These differences limit, to some extent, use of laboratory observations as an aid in interpreting conditions in the field. In these studies the only measurable mortality was that most closely associated with the availability of food. Losses due to predation, probably the most important sources of mortality in nature (5), were not involved at all here. It has frequently been suggested that the most likely victims of predation in nature might be individual larvae that have been weakened by the effects of starvation (5, 11, 15).

Experimental groups in this study which received food were fed to excess. Therefore, the difference in growth attainment between starved and fed groups was probably at a maximum. Under conditions of restricted prey density, even larvae fed early in development might not have enjoyed as great a growth rate. At the same time, satisfactory food is probably never totally absent in nature as it was among the starved groups in this study.

Artemia nauplii are a frequently used laboratory diet for the larvae of fish species that appear to require live food. Although *Artemia* nauplii appear to support a satisfactory rate of growth in laboratory populations, there is little nutritional information available to serve as a basis for comparison between *Artemia* and the variety of micro-crustacea that comprise the natural food of striped bass larvae (27).

In nature, striped bass larvae are present on their estuarine nursery grounds during the spring under conditions of rapidly rising water temperatures. An average temperature rise of 1°C per week is typical in the Hudson River estuary during the period of larval striped bass abundance (36). Constant temperatures were used in these laboratory studies.

With these reservations in mind, some statements may still be made about the probable early growth pattern of striped bass larvae under natural conditions. Data presented here indicate that the size and developmental stage of early striped bass larvae of a given chronological age are intimately related to their thermal and nutritional history. In well studied estuaries, the probable temperature history of a group of larvae spawned at a particular time and location may be estimated with some accuracy. However, a basis for determining the nutritional history of a given group of larvae is not readily obtainable. Even where data are available on the spacial distribution and density of potential food organisms, the frequency with which larvae actually encounter suitable prey can never be known with any degree of accuracy (11).

In assessing the effects of power generating plants on striped bass populations, it is necessary to estimate the rates of natural and plant induced mortality among the pelagic larvae. Life-stage duration estimates, coupled with estimates of stage-specific vulnerability to plant entrainment, may be used to determine the extent of losses that may be attributed to the operation of a particular plant.

Larval mortality rates in nature are frequently estimated on the basis of the relative frequency of occurrence of larvae of various presumed age-classes in ichthyoplankton collections made throughout the period of larval abundance in the water column. The results of this study suggest that the occurrence of large numbers of early post yolk sac larvae in such collections may be a reflection of a period of suspended or slowed growth among larvae which are being subjected to heavy competition for the available food. Without some knowledge of hatching time, temperature regime, and feeding history, there appears to be no way that such larvae may be aged accurately on the basis of size and/or structural development alone.

The use of fixed stage duration estimates in predictive models, especially for that stage immediately following yolk absorption, could lead to serious errors in the resulting estimates of stage-to-stage mortality rates.

ACKNOWLEDGEMENTS

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THE EVOLUTION OF THE BUGSYSTEM: RECENT PROGRESS IN THE ANALYSIS OF BIO-BEHAVIORAL DATA

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ABSTRACT

Experimental investigation of the movements of organisms often entails the acquisition and processing of large samples of spatio-temporal data. An interactive, interpretive, on-line computer-television system (viz., the Bugsystem) was developed in order to expedite such analyses. Aspects of the structure of this prototype system are outlined. Its effectiveness is evaluated with regard to the problems confronting the bio-behavioral researcher.

A second generation system has been developed under a research grant from the Environmental Protection Agency. Utilizing new hardware and software, it in many ways constitutes a *generalization* of its prototype. We describe features of the refined system which provide for the following: a large degree of machine-independence, significant expansion of the size of data records, inclusion of experimental parameters and variables within the data structure, investigation of rotational and flectional movement, statistical analysis, and tracking of organisms in three dimensional space. Current utilization of the Bugsystem for research in behavioral physiology and the potential for future development are discussed.

INTRODUCTION

The fundamental focus of behavioral research is the description and explication of what individual organisms *do*. Because those biological activities most often classified as "behavior" consist largely (although not exclusively) of the movements of organisms, quantitative investigation of behavior is often dependent upon the collection of temporal sequences of spatial information. Cinematography is the classical method used to gather such information. Behavioral variables such as the position of an organism, its orientation, and angles of flection of its appendages are extracted from the motion pictures by means of frame-by-frame analysis. An important advantage of this technique is its flexibility; it may, in principle, be employed in the investigation of any overt behavior which can be photographed. However, two factors preclude the widespread use of this method: (1) manual quantitization via frame-by-frame analysis is a lengthy and tedious process; and (2) once the data is obtained, a substantial amount of subsequent data processing may be required in its analysis.

The Bugsystem (2, 4, 5) was designed to enable the acquisition and processing of video data by a minicomputer. Behavioral data are initially recorded using standard closed-circuit television equipment. These data are analyzed by replaying a video tape into a specially designed video-to-digital processor (christened the "Bugwatcher"). This device acts as an edge detector, greatly reducing the information flow to the computer and thereby allowing the real-time collection of spatial coordinates delineating the outlines of moving organisms. Frame-by-frame analysis of digitized video data is achieved through the use of specially designed programs tailored to the task of quantizing behavioral variables of interest to the researcher.

The original version of the Bugsystem was developed by Greaves and implemented on an IBM 1800 computer at the University of California, Santa Barbara. This prototype system, previously described by Greaves (5), has been utilized by Hand and Schmidt (6) and by Wilson (8) to investigate the photokineses and phototaxes of marine dinoflagellates. However, several features of this system severely limited the domain of its application. Supported by a research grant from the Environmental Protection Agency, we have developed a second generation Bugsystem. Our explicit goal in the design of this system was to provide a *flexible* tool for the quantitative investigation of behavior, a system capable of realizing much of the potential of frame-by-frame analytic techniques.

The purpose of this paper is to describe this second generation Bugsystem, emphasizing the way in which certain hardware and software refinements have expanded the scope of questions which may be conveniently answered by means of "bugwatching." We discuss the way in which the user interacts with the system via a specially formulated "Behavioral Research Language" and the way in which this language has been implemented upon physical machines. We also describe the input of data to the system, the processing and display of behavioral data, and a variety of experimental strategies accommodated by the system. Finally, we outline work now in progress to further generalize the Bugsystem to provide for the analysis of movement in three dimensions.

DEFINING A BEHAVIORAL RESEARCH LANGUAGE

The Behavioral Research Language, or BRL, is a high level operator based language that is tailored to the unique problems associated with the input, scaling, analysis and display of video images of moving objects. BRL is an interpretive language which runs as an application program on a good sized minicomputer and relies heavily upon user interaction with a storage graphics terminal to input, plot, edit and transform the data through image processing functions. Image processing generally culminates in the computation of paths or trajectories of the objects moving before the video camera. Sets of trajectories may be merged together and the data may be transformed to yield time series of behavioral variables (e.g., linear velocity, angular velocity, direction of travel, etc). These results may then be analyzed statistically, and the resultant data sets either listed numerically or plotted on the graphics terminal. Thus, the Bugsystem consists of two basic subsystems: (1) an unique image processing system for the frame-by-frame analysis of video data; and (2) a signal processing system for the statistical analysis of equispaced time series.

The key element to understanding and using BRL lies in grasping the operator-operand-resultant nature of specifying functions or commands to the system. The generai command syntax is as follows:

```
*Operator/sw/sw Operand-name/sw/sw Resultant-name/sw/sw nl, . . . n5,
```

Where "*" is the prompting character, "Operator" is one of the available functions (of which there are currently 88, with the list still growing), "Operand-name" is the name of the input (or operand) data set, and the numeric constants "nl" through "n5" are optional numeric constants which govern details of the function of certain operators. The "/sw" denote optional "switches" (the word is taken from minicomputer jargon) which are used as operator modifiers or to supply special information to the operator being used. All data sets are disk resident and are specified by a four-letter name that can be used to denote an experimental condition, a two-letter extention that specifies the type of data represented (e.g., "VI"-video, "PA"-path, "LV"-linear velocity, "CA"-catergorized, etc.), and a six letter front name that can be used to identify the species studies and/or the date of the experiment. The front name must be specified only when starting the system and remains unchanged unless it is explicitly modified by the **LOAD** operator. Operator names will henceforth be in bold upper case letters in the text.

For example, to plot all of the data in the video file called **BUGS.VI**, the command would appear as "***PLOT** BUGS.VI". To plot only the first twenty frames of this file, one would enter "***PLOT** BUGS.VI 1, 20". To calculate the linear velocities for the paths in the file **BUGS.PA**, the command line is "*LVEL BUGS.PA BUGS.LV". Note that in this language the loops required to access all data elements within a data set are not explicitly stated. The operator automatically processes all of the elements of the operand data file unless directed to a particular subset (e.g., "***PLOT** 1, 20", as illustrated above).

Three other aspects of BRL are worth including here. The first of these concerns the way in which data are represented within the Bugsystem: a data set consists of one or more vectors of variable length. While performing image processing operations, each vector represents one video frame; one element of such a vector represents a single point in two dimensional space. As the analysis of the data proceeds through successive application of operators to operand data sets, a single vector may represent an organism's path (i.e., a time series of cartesian coordinates in two space as in the file "BUGS.PA" illustrated above) or a real function defined over the length of such a path (e.g., the estimate of instantaneous linear velocity as previously illustrated by the file "BUGS.LV"). Finally, in the statistical analysis of such data a vector may correspond to a set of statistical parameters, a collection of "bins" or categories established for histograming, an estimate of an autocorrelation function, etc.

The second aspect of the language to be considered here are the self-documenting aspects of BRL. No one can be expected to memorize all of the 88+ operator names, what they do in detail and the various switches and numeric constants which they expect. To help in this regard, the **NAMES** operator lists on the terminal the names of all the keyboard operators. Moreover, entering "*Operator/HELP" for any of the available operators will cause the system to print a full page of information describing what the operator does, the types of operands for which the operation is defined, what constants and switches are expected and an example of the operator's use.

The third aspect of BRL to be considered involves the construction of user programs. BRL was designed primarily to be an interactive language: the user normally enters commands at the terminal one at a time and thereby directs the analysis to its desired end. It is also possible to create a disk file of commands consisting of operators and operand specifications and to direct the system to execute this stored sequence of operations (a computer program written in BRL) via the **USER** operator.

THE IMPLEMENTATION OF BRL

The implementation of BRL by means of the second generation Bugsystem differs significantly (both in hardware and in software) from that of the prototype system. This section outlines the main features of the new system, giving reasons for their importance.

A New Bugwatcher

The Bugwatcher hardware was redesigned to utilize medium scale integration (MSI) circuitry. Algorithmic state machine (ASM) charts were employed to formulate and to document the new design. Functionally, the new Bugwatcher is similar to its earlier counterpart: it extracts digital X-Y coordinate pairs representing points belonging to image outlines from the video raster scan and stuffs these coordinates into the computer's direct memory access (DMA) channel. The computer program to input these data buffers them and writes them to disk, thereby allowing the collection of extremely long data records. Within such a record each frame of video data is represented as a vector. Each video vector sent by the Bugwatcher to the computer contains not only a variable length list of coordinate data, but also includes a leading header of fixed length. The elements of this header are referred to as "vector attributes" and are employed to encode information associated with each vector. Video data possess four 16-bit words of attribute information supplied by the Bugwatcher hardware: (1) a unique word consisting entirely of zeroes used by the software to delimit frame boundaries; (2) a descriptor word which contains an encoding of the frame rate at which the video data were digitized and the on-off status of tone stimulus markers; (3) a total frame counter that can be used to determine relative or absolute time intervals between data segments recorded at varying time intervals; and (4) auxiliary digital input which allows an encoding of an experimental variable (e.g., the direction of the source of stimulation) to be automatically associated with each video frame. Representation of stimulus conditions is discussed in more detail below (see "Coupling to Research Environments"). This division of vectors into attributes and data applies to all vectors manipulated by Bugsystem software. However the meaning of each attribute depends on the type of data; e.g., one of the attributes of a path (represented as a single vector) is the starting frame number.

The Implementation Language—FORTRAN IV

The prototype BRL system was implemented entirely in assembler language on an IBM 1800 computer and ran as a stand-alone system with no operating system support. This made it non-portable and difficult to maintain and expand. The new BRL system was to overcome these major shortcomings;

thus, we chose FORTRAN IV as the language with which to implement the Bugsystem. FORTRAN IV has become a standard language among minicomputers and has simplified the tasks of maintaining Bugsysem software and training new programmers to implement new BRL operators. The importance of these aspects cannot be overestimated since, all in all, ten different programmers have added software to the system during its three years of development, each requiring instruction on the software conventions and use of system utility subrouting packages. But at least they knew the FORTRAN language.

The software was first operational on a PDP 11/45 computer at Southeastern Massachusetts University under the DOS-9 operating system. It was then implemented on a Data General ECLIPSE S/200 under the RDOS operating system. Some assembler language subroutines had to be recoded, including the software drivers that handle the direct memory channel to the Bugwatcher. But the transition to the new computer went fairly smoothly. The PDP 11 system was maintained for development purposes after the ECLIPSE was sent to its Narragansett home at the EPA lab. Due to malfunctioning DOS-9 software, the PDP 11 operating system was changed to RT 11–a change that required as much or more development effort than changing computers!

Virtual File Structure

Certainly one of the major disadvantages to using minicomputers for large application software projects is the address space limitation imposed by its small word size. This system was no exception. To overcome this limitation so as to allow both programs and data to fit within allocated memory, both program structures and data structures were designed so that only pieces of either resided in memory at any given time. Manufacturers of minicomputers recognize this problem and provide software support for manipulating overlayed programs; i.e., programs consisting of parts which are swapped in and out of main memory. However, software support was not available for similarly overlaying data sets. The earlier prototype system did not allow data sets to be any larger than the memory buffer on the IBM 1800--a simple solution, but not acceptable in the newer system. Certain Bugsystem applications require the acquisition and analysis of behavioral records consisting of many frames of video data or the trajectories of hundreds of organisms. The system required the potential to manipulate data structures ten to hundreds of times the size of main memory available for data.

One of the early software design goals was to simplify the process of adding new BRL operators. Each operator was to be implemented in FORTRAN as a program overlay. An operator would be required to access as many as three simultaneous data sets (e.g., the arithmetic operators **PLUS**, **SUBTRACT**, **MULTIPLY** and **DIVIDE** each require two operands and generate one resultant) within a labeled common buffer of 8192 integer values (or 4096 single precision values). Within the code that implements a new operator, files are accessed with a complete set of virtual file handlers coded in FORTRAN. These routines provide services for opening an existing file, creating a new file, reading a vector into the buffer from disk, writing a vector from the buffer to the disk and closing a file. To minimize disk access time and thereby insure optimum response time to the user, techniques employed in managing other virtual memory systems were adapted to the Bugsystem. All data sets, stored on disk as contiguous files, are accessed directly using multiple block transfers. If, for example, a command is issued from an applications program to read a given vector within a file, then the software first determines if the vector is resident within the buffer. If it is resident, then the routine immediately returns to the calling program providing the length of the vetor and a pointer into the buffer to the first element of the vector. If it is not resident, its logical address within the file is computed and it–and the vectors which succeed it–are read into the labeled common area. A similar algorithm is employed when writing a vector into a file; i.e., a disk write is not required unless the output buffer area is full.

Funneling all file input/output through a common set of routines has significant advantages. The development of new BRL operators is simplified insofar as the underlying applications programs do not each separately (and redundantly) require the algorithms needed to manipulate large files. Another advantage lies in the increased portability of the software. Versions of FORTRAN supported by different machines and operating systems vary most markedly in their non-standardized methods of accessing files. Machine and operating system dependencies are thus isolated in a manageable number of software modules, yielding more portable and more easily maintainable software.

COUPLING TO RESEARCH ENVIRONMENTS

Images

Primary input to the Bugsystem consists of digitized video data. If the video images are sufficiently "clean" (i.e., possess high contrast and lack structural complexity), input of data to the computer is accomplished automatically in real time. Video tapes are replayed into the Bugwatcher which compares the incoming video signal to a "video threshold" set by the user. Those points within the image where the video signal crosses the video threshold are displayed on a video monitor. The user adjusts the video threshold to make these points coincide with the outlines of moving organisms and selects a frame rate (ranging from sixty frames/sec to one frame/min) appropriate for the relative speed of the organisms. When the **BUGWATCHER INPUT** operator is executed, the computer accepts digitized image information from the Bugwatcher at the selected frame rate. Each threshold point is represented in Cartesian coordinates with 8-bit resolution for each of two orthogonal components. A video frame is represented within the resultant data structure as a data vector with a variable number of such points as its data elements. An entire record (or "video file") consists of a temporally ordered sequence of such vectors.

The Bugsystem was originally developed for the investigation of the behavior of motile microorganisms (2). In this application, the organisms are viewed under dark-field illumination swimming within a well slide upon the stage of a compound microscope. However, automatic digitization of data is possible for any study of moving objects for which "clean" video records are available. One of us (Wilson) is currently using the second generation system to study the effects of plane polarized light upon the behavior of aquatic arthropods. The animals move freely within a cylindrical aquarium (diameter ≈ 20 cm) under bright-field illumination and are viewed using a macro lens attached to the television camera. Use of video tape as a storage medium allows experiments to be conducted in a laboratory remote from the site at which the data are analyzed.

Occasionally video recordings are not "clean" enough to allow fully automated digitization (e.g., data collected in the field) or the digitized images are too crude to provide information about details of an organism's anatomical structure (e.g., the orientation of its eyes). A technique has been developed to expedite manual analysis of such data. Using the **PICK** operators, the video tape is examined frame-by-frame. The user selects points upon the screen of a video monitor using a video cursor controlled by a JOYSTICK. A synthetic video signal representing a tiny bright dot is sent to the Bugwatcher which, in turn, sends digitized video information to the computer. Because averaging algorithms are employed, this method affords higher spatial resolution than does fully automated input: each coordinate in the resultant data structure may have 8-9 significant bits in comparison to the 7-8 bits of the normally digitized input data.

Input of video data to the computer effects a mapping of the image as seen upon a television screen onto a two dimensional representational space. Distances within this space ("Bugspace") are measured in arbitrary internal units (or "Bugwatcher units") and, therefore, must be scaled. Using the **LIVE INPUT** operator, the Bugwatcher processed image of a ruler (or any object of known length)–recorded under the same conditions used in gathering behavioral data–is displayed on the screen of a CRT terminal and two points are selected using the terminal cursors. Thus, the ratio between the distances separating the points in Bugspace and in physical space yields a factor or proportionality between Bugwatcher units and conventional units of spatial measurement (e.g., μm, mm, cm, etc.).

Experimental Parameters and Experimental Variables

Biological interpretation of behavioral data requires that the behavior of organisms be related to experimental conditions prevailing at the time of observation. Consequently, we have developed methods to associate experimental parameters and experimental variables with sets of data. An experimental parameter is a quantity characterizing a condition which is constant throughout any given record but may vary from record to record (e.g., temperature, concentration of a pollutant, etc.). Using the **PARAMETER** operator, such numerical constants may be inserted into the set of attributes belonging to each data vector. Parameters may be deleted, modified or listed. They may be used to organize graphical displays (e.g., average linear velocity as a function of temperature) or to modify the execution of certain operations.

An experimental variable is a quantity whose value changes during a single record. Times at which simple step changes in stimulus conditions (e.g., switching a light on and off) occur may be indicated by the presence or absence of tones stored on the audio track of a video tape. The Bugwatcher possesses four tone generators to produce such temporal markers during the course of an experiment; it also possesses external connections which allow the simultaneous gating of laboratory apparatus. When the video tape is replayed into the Bugwatcher these tones are detected. As discussed above, the second attribute in each data vector sent to the computer contains four bits dedicated to representing the presence or absence of the four tones.

We are presently developing a technique which provides for the representation of stimuli which vary continuously over time. The stimulus level will be encoded by means of frequency modulation (fm) on the audio track of the video tape. When the tape is replayed into the Bugwatcher the fm signal will be digitized and represented with 10-bit precision by the fourth attribute of each data vector sent to the computer. The **APPEND** operator will be employed to extract this information from each data vector, scale it and store as an equispaced time series. Again these data may be used to organize graphical displays or they may enter into computations involving time series of behavioral variables. One of us (Wilson) is preparing to employ this method to investigate behavior evoked by rotation of the plane of polarized light. The organisms swim beneath a polaroid filter whose angle is controlled by an analog servomechanism. The filter may be rotated so as to describe quick jumps, ramps, harmonics, etc. A signal directly proportional to the angle of the filter will be encoded and analyzed.

PROCESSING AND DISPLAY

Image Processing

As discussed above, input of video data to the computer entails substantial preprocessing of pictorial information. A data vector within a resultant video file is a list of contemporaneous points; an organism's outline is represented within this data structure as a localized set of points. The user can display such data graphically (using the **PLOT** operator) or alpha-numerically (using the **LIST** or **EXAMINE** operators). Video files may be edited both in time and in space. The **EDIT** operator allows one to save (or delete) temporally contiguous sets of data vectors. Thus, the user could **EXAMINE** the data to ascertain the frame at which the status of a tone had changed (indicating a change in stimulus conditions, e.g., switching on a blue light) and then **EDIT** the data to insure that this change occurs on frame number 100. The **MASK** operator allows one to save (or delete) points within rectangular or circular regions of the image plane. Thus, the user could **MASK** out all points within a video file which correspond to a particle of detritus within the experimental preparation. Finally, the user may **APPEND** additional information to a video file (text describing the conditions of the experiment, numerical constants, time series of tone states or time series of experimental variables).

Analysis of video data by means of the Bugsystem proceeds by abstracting one (or more) points from each point set delineating an organism's outline. In an investigation of translational movement this task is easily defined: unlike either rotational or flectional movement, quantitative description of the translational component of an individual organism's behavior does not require detailed knowledge of the organism's external anatomy. The body of the organism is represented by a single point, viz., its "center of mass". Translational movement is defined as displacement of this point from one position in space to another.

Mapping outlines into centrally located points is usually achieved by means of the **CENTROID** operator whose command syntax is exemplified by the entry

```
*CENT BUGS.VI BUGS.CE N1, N2, N3.
```

Each vector in the resultant file "BUGS.CE" corresponds to a vector in the operand file "BUGS.VI". Each element of a resultant data vector is a "centroid": a point in Bugspace whose X and Y coordinates are, respectively, the average X and Y coordinates of an "acceptable set" of points in the corresponding operand data vector. The numerical parameters "n1", "n2" and "n3" are required to characterize an "acceptable set" of operand data points.

An initial member (having non-zero coordinates) of such a set is chosen from the operand data vector, the coordinates of this point are set equal to zero in the operand data and a mask (width = 2·n1, and height = 2·n2) is centered upon this point. The set is augmented if an operand point (having non-zero coordinates) falls within the mask. The mask is then centered upon the new point and the search continues. The search terminates when the set is deemed "acceptable" if it possesses at least n3 members. Ideally, this process yields one centroid corresponding to each outline—unless the outlines of two organisms are merged. The user can **PLOT** the centroids over the original data to confirm this correspondence and recompute the centroids using new parameters if the correspondence is not adequate. The structure of "centroid data" is quite similar to that of video data except that each coordinate of a centroid is represented with 15-bit (rather than 8-bit) precision in attempt to exploit the greater accuracy resulting from the averaging procedure. Centroid data is displayed and edited in the same fashion as video data.

The **PICK** operator provides an alternative method to abstract points of interest from video data. The use of this operator for direct analysis of a videotape, thereby, bypassing the acquisition and processing of video files, is discussed above (see "COUPLING TO RESEARCH ENVIRONMENTS"). The user may also **PICK** points associated with each outline by means of the terminal cursors. The **PICK** operator is indispensable for investigations of rotational and flectional movement. For example, the user might elect to study the orientations of the longitudinal body axes of a group of organisms (i.e., simple rotation in a single plane). The longitudinal axis may be defined as a vector extending from the tail to the head of an organism. Thus, the user selects an ordered pair of points corresponding to each outline by entering

```
*PICK/VI BUGS.VI BUGS.TH
```

and using the cursors to specify first the "tail" and then the "head" associated with each outline. The resultant file "BUGS.TH" possesses the structure of centroid data with each "tail point" immediately followed by the correlated "head point". These points are then segregated using the **PLUCK** operator. For example, the two commands

```
*PLUC BUGS.TH BUGS.T 1, 2
*PLUC BUGS.TH BUTS.H 2, 2
```

respectively produce the files "BUGS.T"—containing all first elements of the ordered pairs (viz., the "tail points)—and "BUGS.H"—containing all second elements of the ordered pairs (viz., the "head points). This type of analysis may be extended to encompass larger collections of points (or n-tuples), thereby providing for the study of flectional movement (e.g., the angles of propulsive appendages with respect to the longitudinal axis).

Regardless of the type of movement under investigation, the next stage of image processing entails the computation of paths or trajectories through Bugspace. A path or trajectory is a time-ordered set of points represented as a single data vector and characterized as follows: All points in a path are selected from an operand file consisting of centroids or having the structure of centroid data. The manner in which points are represented in a path is identical to the way they are represented in a frame of centroid data. Each path starts within a specific frame and its starting frame number is represented as the fourth attribute of the vector. Over the temporal interval during which a path is defined one (and only one) point is selected from each corresponding vector (or frame) of the operand file. Adjacent pairs of points within a path are selected on the basis of their spatial contiguity within adjacent frames.

The search for paths is performed by the **PATH** operator whose command syntax is exemplified by the entry

PATH BUGS.CE BUGS.PA n1, n2, n3, n4, n5.

The numerical parameters "n1" through "n5" control various details of the search. n1 specifies the width (in Bugwatcher units) of a square mask used as a criterion of spatial contiguity. n2 is the maximum number of times to expand the mask if no contiguous point is located within the frame being searched. n3 is the n4 minimum number of points to be accepted as a valid path. n4 is the minimum average displacement in Bugspace between consecutive frames for a set of points to be accepted as a valid path; this parameter may be used to "weed out" stationary artifacts (e.g., a particle of detritus). n5 is the maximum number of frames to "look ahead"; i.e., if no contiguous point is found in the current frame, then the next frame may be searched. If a point within the new frame qualifies, the missing point is computed by linear interpolation.

Ideally, every path would correspond to the movement of a single point (associated with the outline of one individual organism) through Bugspace and each such movement would be represented by one path. In practice, the **PATH** operator may commit two types of errors. The operator may overlook certain segments of continuous movement. Such omissions may yield an abnormally short path, or they may result in a one-to-many (even a one-to-none) correspondence between real word trajectories and paths. Alternatively, the operator may confound certain segments of the continuous movements of two (or more) organisms. If there were but one organism in the field of view at any time, then errors of omission could be abolished by using a large mask (why not let it include all of Bugspace?) and allowing the program to look ahead several frames. But with several organisms represented within each frame there is clearly a tradeoff in choosing parameters so as to reduce the incidence of the two types of errors. The user may **PLOT** the paths over the centroids (or over the original video data) to check their fit and then recompute the paths using new parameters. Often several iterations of this process are required to obtain an optimum combination of parameters. Since the parameters depend largely upon the magnification, the density of organisms and the way in which they move, the same "optimum set" of parameters (retained in memory for the user's convenience) are generally used to compute paths for all replicates of an experiment.

Proper selection of pathfinding parameters can significantly reduce the number of erroneous paths but cannot be relied upon to eliminate all errors. Consequently, we have developed programs which allow the user to interatively detect and correct mistakes within path files. Path editing programs (including **CHOZ**, **EDIT**, **MERG** and **JOIN**) allow the user to preform the following basic operations: (1) delete a path; (2) truncate a path; (3) cut a path into two smaller paths; and (4) join two paths (assuming they do not overlap in time).

Once a valid collection of paths has been obtained, the user may proceed directly to the extraction of time series of behavioral variables (as discussed below) from the path files. Before doing so, however, there are several additional procedures which the user may choose to apply to the path data. Since the use of these procedures (and the order in which they are applied) is dependent upon the overall design of the experiment, we will first illustrate them by means of a specific example.

Wilson video taped the behavior of *Daphnia pulex* (a small freshwater crustacean, commonly known as a "water flea") in Talbot Waterman's laboratory at Yale University. Twelve animals were observed from below swimming against a brightly and uniformly illuminated background. A variable polarizer/depolarizer was interposed between the chamber containing the animals and the light source. The tape consisted of 23 separate video records. Before each recording the polarizing filter was rotated to a randomly chosen angle and, also in random sequence, the device was adjusted so as to polarize or depolarize the illumination. Each record began with the image of a strip of plastic attached to the filter in order to indicate the angle of the filter and the magnification of the image. The plastic strip was then removed and the animals were observed swimming under constant conditions. In all, 13 records were obtained under polarized light and 10 records were obtained under unpolarized light. Each recording lasted two minutes.

The video tape was analyzed with the aid of the ECLIPSE at Narragansett, R.I. For each record the **LIVE INPUT** operator was used to determine the angle of the filter with respect to the Bugsystem reference frame (which is fixed with respect to the raster scan of the video signal). As discussed above, the appropriate spatial scale factor (approximately 0.40 mm/Bugwatcher unit) was also computed. The **BUGWATCHER INPUT** operator was then used to generate one video file (480 frames of data at ten frames/sec. or 48 sec. of data) for each record on the tape. Wilson then **MASK**ed the data, saving only those points within a centered circular region of Bugspace. The centroids and then the paths were separately computed for each masked video file using the same set of centroid and pathfinding parameters. Very few pathfinding errors resulted from these computations, but these were corrected by editing the separate path files. Wilson then employed the **PARAMETER** operator to associate the angle of the filter with respect to the Bugsystem reference frame (an experimental parameter) with each path. This angle was the same for every path within a given path file. By repeated use of the **MERGE** operator, Wilson condensed the data to produce two exceedingly large files: one containing all paths observed under polarized light (154 paths, 13210 data points) and another containing all paths observed under unpolarized light (166 paths, 13691 data points). These paths were still represented in Cartesian coordinates relative to the Bugsystem reference frame. However, orientation with respect to the plane of polarization should only be manifest with respect to the reference frame of the filter. Using the **ROTATE** operator, the two frames of reference were made to coincide: every path within each merged file was rotated in Bugspace (about an axis passing through the center of this space) through an angle obtained by negating the appropriate experimental parameter for each path. All resultant paths for *Daphnia* swimming under polarized light are **PLOT**ed in Figure 17-1. Many of these paths may be seen to be aligned approximately orthogonal to the axis of the filter (i.e., perpendicular to the E-vector of the polarized light).

The preceding analysis illustrates the use of three operators (vis., the **PARAMETER**, **MERGE** and **ROTATE** operators) to organize path data *prior to* the computation of behavioral variables. The **ROTATE** operator was implemented to expedite studies of animal orientation. Using this operator, all spatial data in a file may be rotated through a constant angle, each path may be rotated through a constant angle associated with that path or each point may be rotated through an angle associated with a corresponding moment in time. The last option enables the investigator to study orientation with respect to a moving stimulus (e.g., be **MERGED**) whenever the respective files may be taken to be replicates of the *same* experiment. Not only does this simplify the bookkeeping tasks associated with subsequent analysis, but, in addition, allows a set of similar data to be treated as a single sample by statistical operators. The fundamental advantage of an operator-based interactive system for the analysis of behavioral data is its flexibility: the operators which the user chooses to apply to the data—and the order in which they are applied—can be selected to correspond to the design of the original experiment.

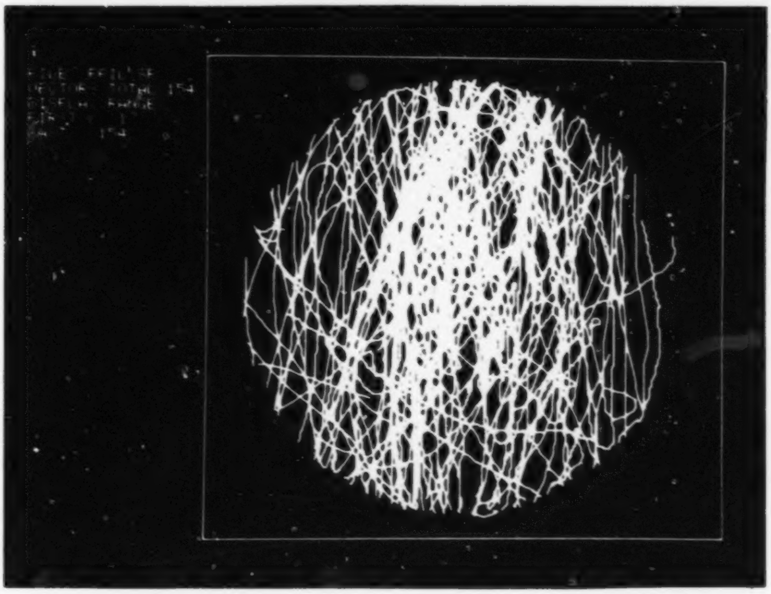

Figure 17-1. Merged Path File of Daphnia Pulex Swimming with Respect to the Reference Frame of the Linear Polarizer.

NOTE: The E-vector is horizontal to the field of view. The experiments and analyses which gave rise to these data are discussed in the text.

Generating and Transforming Time Series Data

Time series of X and Y coordinate values are generated from path data by my means of the **SPLIT** operator whose command syntax is illustrated by the entry

```
*SPLI BUGS.PA BUGS.X BUGS.Y.
```

Each data vector in the resultant files "BUGS.X" and "BUGS.Y" is, respectively, a time series of X and Y coordinate values. The resultant data sets are in one-to-one correspondence and each element is represented as a single precision floating point number. Like the paths from which they are derived, resultant time series may start and end at arbitrary points in time. Therefore, the number of series defined at any given moment is also arbitrary. Other computer systems have been developed for the analysis of equispaced time series (7). After image processing has been completed, the operators available on the Bugsystem are unique only insofar as they possess the sophistication required to manage large collections of arbitrarily derived series (e.g., Figure 17-2).

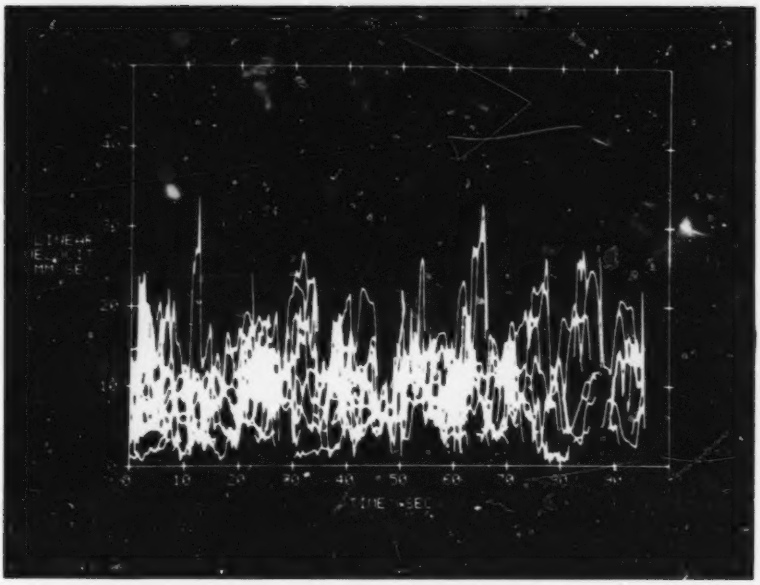

Figure 17-2. Linear Velocity as a Function of Time for 128 Paths of the Fairy Shrimp Eubranchipus Vernalis in Polarized Light.

In principle, all behavioral variables which may be investigated using the Bugsystem can be generated by simple arithmetic transformation of series of X and Y coordinates. Greaves (4) has discussed the computation of certain behavioral variables (viz., linear velocity, net to gross displacement ratio, direction of travel and angular velocity) using arithematic operators implemented within the prototype system. Such operators transform every element of every data vector within a file, treating each vector as a separate unit of data. The present Bugsystem is also provided with a wide selection of simple arithematic operators. However, we have condensed the computation of certain frequently calculated behavioral variables into single operators which require path data as input. As an example, let us consider the **RATE OF CHANGE OF DIRECTION** operator whose command syntax is illustrated by the entry

```
*RCDI BUGS.PA GUBS.RD n1.
```

Every element in the resultant file "BUGS.RD" is the unsigned rate at which the corresponding path changed its direction of travel at a given moment. RCD is the absolute value of angular velocity expressed in degrees per second (The parameter "n1" is the frame rate, required to convert from degrees per frame.) The importance of this variable in ascertaining mechanisms responsible for certain animal aggregations has been widely discussed (3). RCD may also be computed by a sequence of simple arithematic operations as follows:

```
(a) *SPLIT BUGS.PA BUGS.X BUGS.Y
(b) *CDIF BUGS.X BUGS.DX
(c) *CDIF BUGS.Y BUGS.DY
(d) UCTAN BUGS.DX BUGS.DY BUGS.DI
(e) *CDIF BUGS.DI BUGS.AC
(f) *MULT/CO BUGS.AC BUGS.AV nl
(g) *ABSV BUGS.AV BUGS.RD
```

Where "**CDIF**" is the **CENTRAL DIFFERENCE** operator (a discrete approximation to the differential operator), "**CTAN**" is the **CONTINUOUS** ARCTANGENT operator, "**MULT/CO**" denotes multiplication by a constant and "**ABSV**" is the **ABSOLUTE VALUE** operator. The resultant "BUGS.DI" of step (d) is the direction of travel measured in degrees with respect to the Bugsystem reference frame; it could have been generated from the original path data using the **DIRECTION OF TRAVEL** operator. The resultant "BUGS.AV" of step (f) contains angular velocities (measured in degrees per second); this file could have been produced using the **ANGULAR VELOCITY** operator. Other operators have been developed to evaluate **LINEAR VELOCITY** and **NET TO GROSS DISPLACEMENT RATIO** functions defined upon path data.

Simple Statistical Processing

For the purpose of statistical analysis two different types of data structure–representing two levels in a structural hierarchy–may be distinguished as "samples": vectors and files. Many statistical operators recognize this distinction. For example the **STATISTICAL PARAMETER** operator estimates parameters such as the mean, variance, standard deviation, skewness, kurtosis, etc. The command

```
*STAT/VE BUGS.LV BUGS.ST
```

produces the resultant file "BUGS.ST", containing one data vector (i.e., a list of statistical parameters) for each data vector in the operand, whereas the command

```
*STAT/FI BUGS.LV BUGS.ST
```

produces only one resultant data vector characterizing the entire file. In either case, the user can **LIST** the resultant parameters. Similarly, the **SLOT** operator provides for estimation of density and distribution functions via histograms both for individual vectors and entire files. These data may be displayed graphically (Figure 17-3) or **LIST**ed on the terminal or the line printer.

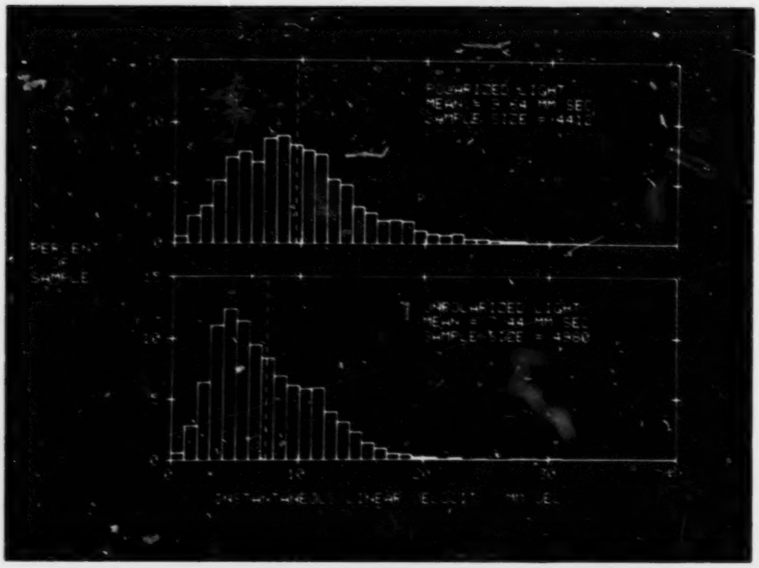

Figure 17-3. Linear Velocity Histograms for E. Vernalis in Polarized Light (the Data Displayed in Figure 17-2) and in Unpolarized Light.

NOTE: Dashed lines denote the estimated mean of each distribution.

We chose to store statistical parameters within data vectors, rather than merely computing and displaying them, in order to allow them to enter into subsequent calculations. For example, assume the user had computed statistical parameters for each data vector in a file of instantaneous linear velocities (e.g., the "**STAT**/VE" example given above); each estimate of the mean is thereby an average for each path. The user may then investigate the distribution of these path averages. The estimated means are first isolated using the **STRIP** operator:

***STRI**/MN BUGS.ST BUGS.MN.

The resultant file "BUGS.MN" contains a single data vector; each element of this vector is a mean ("/MN") stripped from one operand data vector. The user may wish to compute statistical parameters for the new set of data, or explore its frequency distribution via histograms (Figure 17-4). The user may also **MERGE** such files so that each data vector corresponds to a single experimental condition. The vectors may then be compared with one another (e.g., one-way analysis of variance, chi-square tests, etc.).

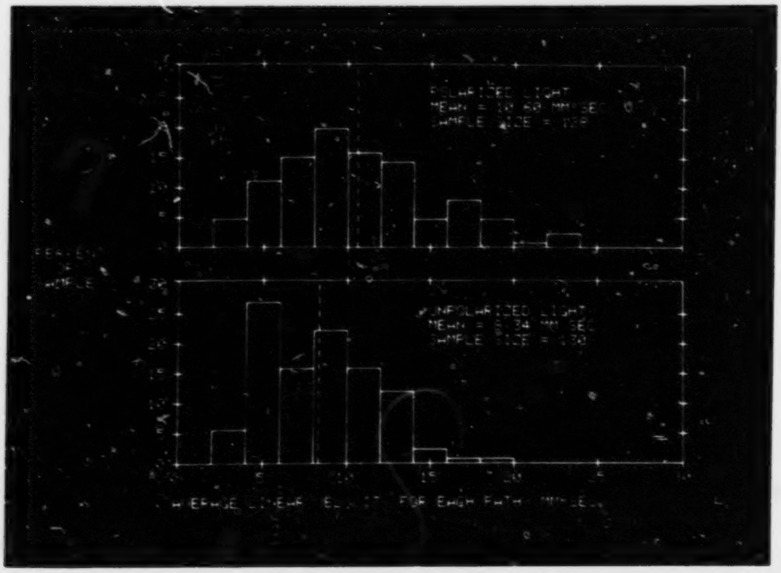

Figure 17-4. Average Linear Velocity Histograms for E. Vernalis in Polarized Light and in Unpolarized Light.

NOTE: Dashed lines denote the estimated mean of each distribution.

We have made special provision within the Bugsystem for the manipulation and statistical analysis of angular data. For example, the command

```
*STAT/AN/FI BUGS.DI BUGS.ST
```

produces a single data vector whose elements are statistical parameters appropriate for circular distributions. These include the length and direction of the mean vector (and related measures) as discussed by Batschelet (1). Moreover, because the Bugsystem is well suited for investigations of animal orientation (and because this is a major interest of one of its codevelopers) we have implemented an extensive polar graphics package within the Bugsystem. Figure 17-5 illustrates the use of polar wedge histograms to represent angular density functions.

More Advanced Statistical Operations

These fall into two major categories: operators directed toward the analysis of time dependence of behavioral variables and operators used to explore mutual relationships between variables (e.g., Figure 17-6). In the first category,

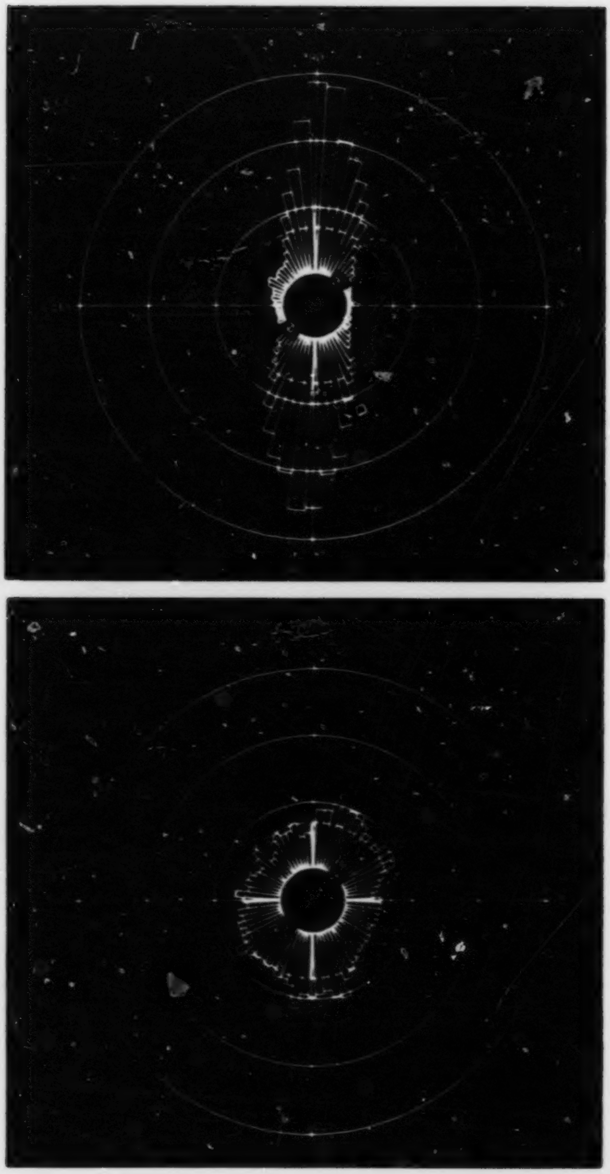

Figure 17-5. Polar Wedge Histograms of Instantaneous Direction of Travel with Respect to the Filter Reference Frame for D. Pulex.

NOTE: (A) in polarized light (evaluated for the path plotted in Figure 17-1) and (B) in unpolarized light. The percent of sample within each angular category is indicated on the radius. The dashed circles denote expectation for a circular uniform distribution.

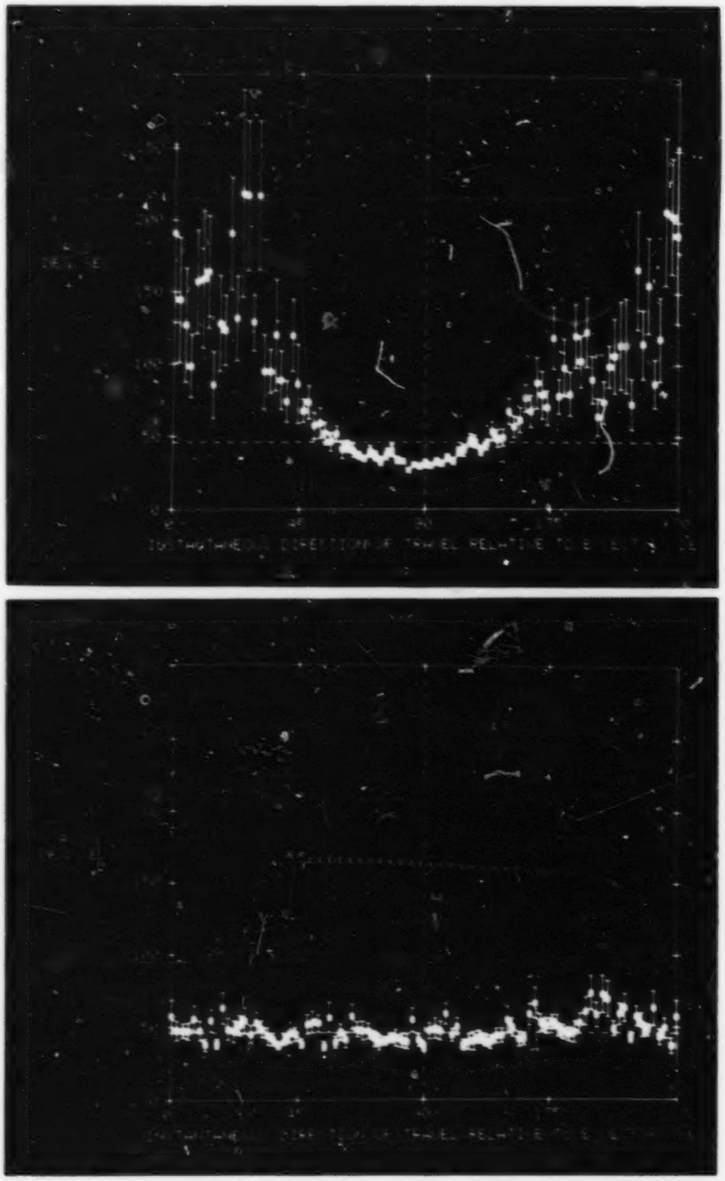

Figure 17-6. Dependence of RCD (Rate of Change of Direction) Upon Direction of Travel with Respect to the Axis of the Polarizer for D. Pulex.

NOTE: (A) in polarized light and (B) in unpolarized light. Data was processed by partitioning RCD into disjoint subsets on the basis of the correlated direction of travel. The estimated mean (± the standard error of the mean) for each subset is indicated in the figures.

the Bugsystem includes several ensemble operators which compute a statistic (e.g., an estimate of the mean or mean vector or even a histogram) for every frame defined within the operand data. The Bugsystem also includes serial correlation operators (viz., **AUTOCORRELATION** and **CROSS CORRELATION**). Programs providing for analysis in the frequency domain, sinusoidal, regression and polynomial regression are currently under development.

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THE EFFECTS OF TEMPERATURE, LIGHT AND EXPOSURE TO SUBLETHAL LEVELS OF COPPER ON THE SWIMMING BEHAVIOR OF BARNACLE NAUPLII

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ABSTRACT

The "Bugsystem" is a computer-television system developed to accurately track and analyze swimming patterns of aquatic organisms. Video images of test animals are converted to time sequence X-Y coordinates to allow rapid computer analysis of linear or angular velocity, rate of change of direction, direction of travel and other parameters. Initial experiments using barnacle nauplii (*Balanus amphitrite, B. improvisus, B. venustus, Chthamalus fragilis*) indicate larval swimming speeds are affected by temperature and light regime. Response to temperature appears to be a function of species tested and, perhaps, geographic location of the adult population. Changes in linear velocity induced by acute light intensity variation are of short duration. Mean linear velocities of nauplii are altered by 24 hour exposure to copper as low as 20 ppb. Linear velocities of exposed populations increase relative to controls at low copper levels, and then decrease as lethal levels are approached. Copper will also alter the swimming pattern of exposed larvae.

INTRODUCTION

In view of concern that bioassays directed solely toward determining lethal concentrations of pollutants may not accurately reflect levels doing harm to the environment, attention has been directed toward sublethal effects of pollutants– "effects which do not immediately, or directly, lead to death, but which nevertheless cause disturbances which may be of ecological significance (1)." Existing studies using pathological, physiological, and behavioral parameters indicate approximate thresholds for sublethal responses are often 10-20 percent of LC50 levels or less (7, 22). It is generally recognized that behavioral responses of marine animals are often highly sensitive to stress (18) and that juvenile or larval stages of many marine organisms represent that part of the life-cycle most susceptible to stress (4, 8, 16). Logically, larval behavioral responses to pollutants would represent a potentially significant field of study; however, devising a means to easily record and rapidly quantify swimming and other responses of small larvae has limited efforts in this direction (8, 12, 17, 24).

Development of the Bugsystem at this laboratory has provided the technology to rapidly analyze the swimming patterns of large sample numbers of organisms of a wide size range (Wilson & Greaves, this volume, report 17). With this potential we are presently investigating the use of behavioral bioassays for marine larvae. The following results represent initial studies using larvae of common barnacle species.

EXPERIMENTAL

The spontaneous locomotory activity for second stage nauplii of four barnacle species (*Balanus amphitrite amphitrite, B. improvisus, B. venustus, Chthamalus fragilis*) was investigated. Of primary concern in this initial study was the mean linear velocity (MLV) of sample groups and changes in MLV induced by water temperature, light regime, and 24 hour exposure to sublethal copper levels.

Source of Larvae

Second stage *Balanus* nauplii used in all experiments were released from adult barnacles maintained at 20 ± 2°C, constant illumination in 30-32°/oo, 1 μ filtered seawater from Narragansett Bay. *Balanus amphitrite* and *B. improvisus* were initially collected near Georgetown, South Carolina, and maintained under the above laboratory conditions 2-12 weeks prior to larval release. *B. venustus* adults were collected in Narragansett Bay; adults released larvae within two weeks after capture. *Chthamalus fragilis* nauplii, also from Narragansett Bay, were obtained from ripe egg masses incubated for 24 hours at 20°C. In some copper experiments, stage II nauplii were reared to later stages on a mixed algal diet of *Tetraselmis suecia* and *Thalassiosira pseudonana*. Methods of maintaining laboratory populations of barnacles and rearing of nauplii are further described by Lang (13, 14).

Video Recording

To obtain video tapes of swimming patterns for Bugwatcher analyses, ca.30 nauplii were placed in 25 ml beakers with a water depth of about 10 mm. The beaker with nauplii was put within a cylindrical flat black metal lenshood (light shield) attached to a 72 mm diameter #25 deep red glass filter (Figure 18-1). This complex was centered upon a Wild M-5 dark field illumination stage fitted with an "800 nm" interference filter and 22 mm diameter diaphram (Figure

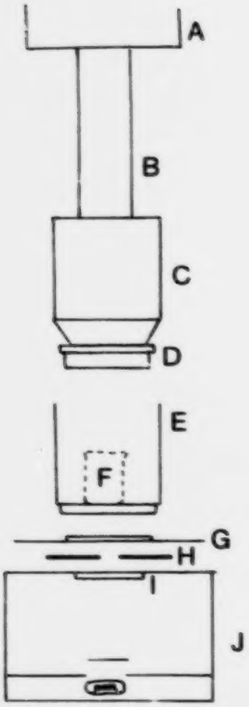


Figure 18-1. Diagram of Video Recording Equipment.

NOTE: A) TV camera, B) Photo tube, C) Wild M-5 microscope, D) wide-angle attachment, E) metal light shield with deep red glass bottom, F) container with test organisms, G) 800 nm interference filter, H) metal diaphram, I) clear glass, J) dark field stage with halogen light. Components E-H are shown separated from each other for graphic clarity.

18-1). Video images were obtained using a Cohu 4400 television camera attached to the M-5 microscope body. The field of view recorded was 10 x 10 mm. Optimum image contrast was obtained using a halogen light source with dark field optics at 8.5 volts (Figure 18-1).

Spectroradiometer (ISCO-SR) readings indicate light transmitted through the stage filters was as low as 680 nm. Peak transmission occurred between 810-840 nm. With the exception of light experiments, nauplii were moved directly from constant light temperature boxes to the microscope stage. Room lights were extinguished and, following a two-minute acclimation period, larvae were taped for 3-5 minute intervals using filtered substage illumination. Swimming parameters reported were determined by analysis of 30-60 second portions of these tapes; results are pooled from replicate samples.

Response to Light

Nauplii used in all experiments were light adapted as fluorescent bulbs in the temperature boxes were on continuously. When transferred to the darkfield stage, larvae tended to disperse to the beaker walls with ceiling lights on. With these lights extinguished, nauplii tended to swim away from the beaker walls. Direction of travel upon entering or leaving the camera field exhibited no particular orientation. Ongoing studies indicate dark adapted *B. amphitrite* nauplii will exhibit a weak photonegative response to substage light over a five-minute period (Forward & Lang, personal observation).

Balanus spp. stage II nauplii exhibit similar response to sudden changes in light intensity. When overhead white room lights are turned on, *Balanus* nauplii will approximately double linear velocities, then within 4-6 seconds return to initial swimming speeds (Figure 18-2). Turning overhead lights off has essentially the opposite effect; nauplii will cease locomotion for about five seconds and then return to initial swimming speeds (Figure 18-3).

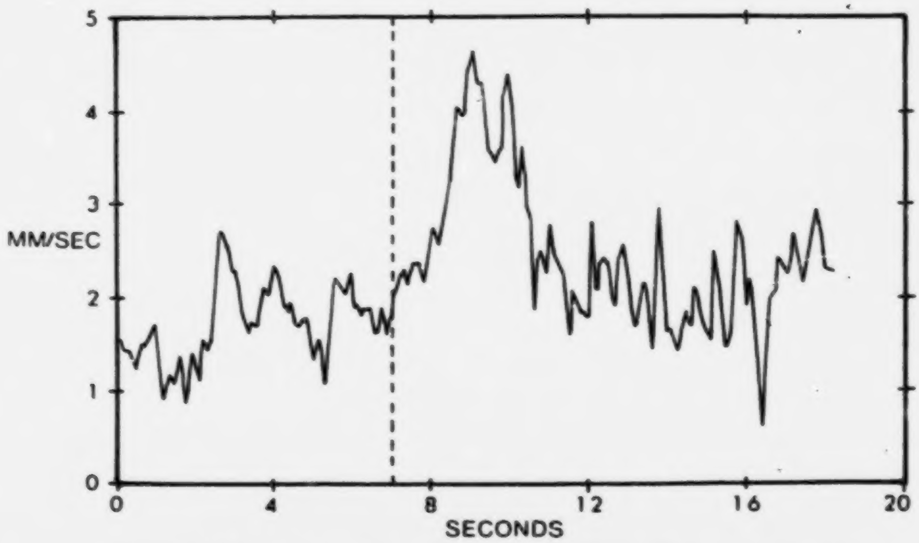


Figure 18-2. Example of running average linear velocity (mm/sec) for sample of ten stage II Balanus venustus nauplii exposed to sudden light increase.

NOTE: Dashed line indicates time at which overhead white light stimulus was applied. Filtered (820 nm peak transmission) substage light was present throughout experiment for recording purposes.

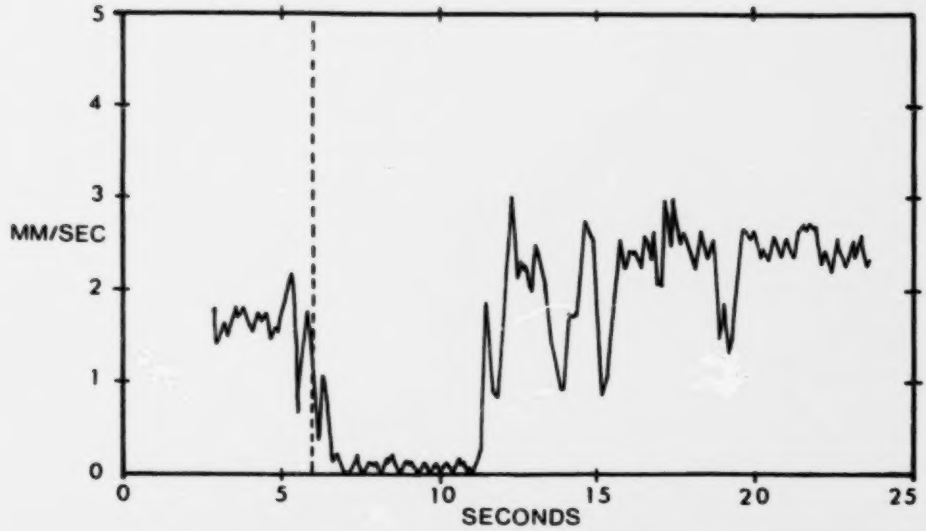


Figure 18-3. Example of reaction of a single stage II Balanus venustus nauplius to sudden light decrease.

NOTE: Dashed line indicates time at which overhead white light was extinguished. Filtered (830 nm peak transmission) substage light was present throughout experiment for recording purposes.

Chthamalus fragilis exposed to similar light changes exhibited little response in terms of MLV. The distinctive response seen with *Balanus* nauplii was clearly absent.

Response to Temperature

Newly hatched nauplii from the same brood (incubated at 20°C) were subdivided and placed into various temperature boxes for 24 hours to test the effects of temperature on swimming velocity (Table 18-1). The metal lightshield, glass filters, and beaker with larvae were equilibrated to the test temperature, then transferred immediately to the microscope stage for brief taping. Readings with a temperature probe indicated a maximum 2°C shift toward ambient occurred during taping.

Even with the potential of a 2°C deviation in test temperatures, certain geographical distinctions are suggested between swimming velocity and temperature (Table 18-1). Data from *Balanus amphitrite* and *B. improvisus* nauplii from South Carolina adults suggest a direct relationship of increased swimming velocity with increased temperature. In contrast, *B. improvisus*

Table 18-1. Mean Linear Velocities (MLV) of Stage II Barnacle Nauplii Exposed to Different Temperatures for 24 Hours.

Species	Temp. (°C)	N	MDP (Sec)	MLV ± sd (mm/sec)	T test
BA	28	12	8.5	0.81 ± 0.23	NS
BA	20	24	9.0	0.71 ± 0.34	
BA	15	22	7.1	0.58 ± 0.20	P=.20
BIS	25	26	7.4	1.18 ± 0.56	NS
BIS	20	26	6.0	1.09 ± 0.43	
BIS	15	25	8.0	0.72 ± 0.43	P=.05
BIN	26	13	7.3	1.56 ± 0.62	P=.10
BIN	22	17	9.4	2.43 ± 0.64	
BIN	10	14	8.0	1.25 ± 0.64	P=.05
CF	25	26	7.2	1.46 ± 0.48	P=.05
CF	20	40	6.4	1.92 ± 0.67	
CF	15	40	6.0	1.89 ± 0.62	NS

nauplii from Rhode Island adults exhibited a decreased velocity above 22°C. A comparable reduction in velocity with Rhode Island nauplii also occurred with *Chthamalus fragilis* above 20°C. Yet overall, naupliar swimming speeds appear to be greater in the Rhode Island animals when measured within this temperature range.

Brood Variability

Linear swimming velocity in nauplii obtained from different broods of South Carolina adults was assessed under similar temperature, salinity, and light regimes to evaluate brood variability. Linear velocities were found to be similar among six broods (Table 18-2). Examples of MLV distributions within test groups of *B. amphitrite, C. fragilis*, and *B. improvisus* are shown in Figure 18-4. Control groups usually have linear velocity distributions which approximate normal or are skewed to the left.

Table 18-2. Mean linear velocites (MLV) of various hatches of stage II Balanus amphitrite nauplii at 20°C.

Hatch No.	N	MDP (sec)	MLV ± sd (mm/sec)
3	40	7.0	0.75 ± 0.34
8	57	5.3	0.72 ± 0.20
9	14	6.1	0.65 ± 0.43
14	24	9.0	0.71 ± 0.23
24	23	7.4	0.72 ± 0.27
25	20	7.0	0.85 ± 0.33

NOTE: N = number of paths analyzed, MDP = mean duration of paths analyzed.

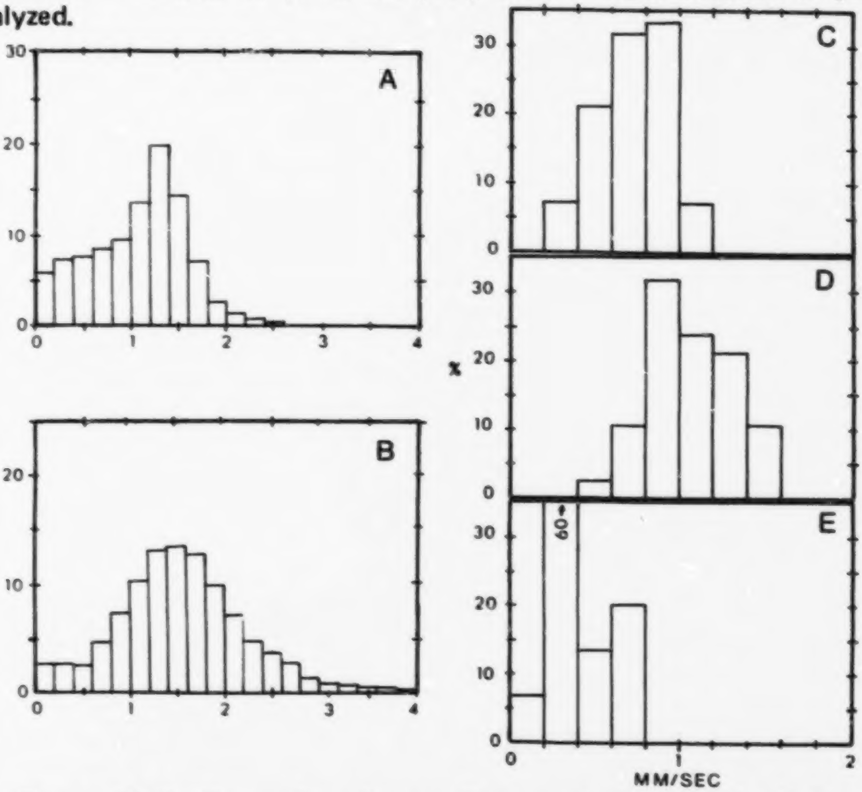


Figure 18-4. Examples of mean linear velocity (mm/sec) distribution within five test groups of stage II barnacle nauplii:

NOTE: A) *Balanus improvisus* from Rhode Island at 20°C, B) *Chthamalus fragilis* at 20°C, C) *Balanus amphitrite* control, D) *B. amphitrite* with 24 hr. exposure to 20 ppb Cu, E) *B. amphitrite* with 24 hr. exposure to 350 ppb Cu.

Response to Copper

Having characterized linear swimming velocities for *B. amphitrite* nauplii under defined conditions, the effects of sublethal levels of copper on swimming speeds were investigated. A primary stock solution of 10,000 ppm Cu^{++} in dilute nitric acid was adjusted to 1-4 ppm secondary stock solutions using deionized water. Final test solutions were obtained by serial dilutions with 1 μ filtered natural seawater at 32-34°/oo. Total copper was determined by heated graphite absorption on a HGA-2100 coupled to a Perkin-Elmer 360 atomic absorption spectrophotometer.

Newly hatched *B. amphitrite* nauplii were exposed to various copper levels for 24 hours at 20°C. Replicate samples of nauplii for each exposure level were then video taped in a darkened room using the described darkfield illumination. Total mortality at 24 hours post-exposure was determined. Larvae were not fed during the experiment. Analyses indicated a 3-15 percent decrease in total copper occurred during a 24-hour static exposure. In the first test, nauplii were exposed to levels ranging from control (3 ppb) to approximately 50 ppb Cu (Table 18-3). No increased mortality was observed; however, MLV of nauplii exposed to 10 through 47 ppb Cu nearly doubled relative to control values. Close agreement was observed in replicate samples.

In a second test, three higher copper levels were added: 120, 185, 350 ppb. After the 24 hour Cu exposure period, nauplii were transferred to clean filtered seawater with the mixed algal diet. Significant mortality at 24 hour post-expossure occurred only at 350 ppb Cu. However, a delay in molting was

Table 18-3. Mean Linear Velocities (MLV) of Balanus Amphitrite Stage II Nauplii Exposed to Sublethal Copper Levels for 24 Hours at 20°C

Copper (ppb)	No. Animals	Mean Path Duration (sec)	MLV ± sd (mm/sec)	Mortality 24 Hr. Post Exposure
3	16	6.4	0.71 ± 0.27	3%
3	14	6.2	0.65 ± 0.44	5%
10	12	5.0	1.24 ± 0.48	4%
12	10	7.3	1.18 ± 0.51	2%
14	21	7.6	1.09 ± 0.54	2%
14	6	7.3	1.06 ± 0.76	4%
30	10	7.8	1.07 ± 0.54	2%
47	15	6.4	1.25 ± 0.47	5%
47	11	7.0	1.16 ± 0.48	5%

seen at 48 hours post-exposure; only 35 percent of nauplii exposed to 185 ppb Cu had molted to Stage III, as opposed to nearly 70 percent at lower copper levels. (Figure 18-5.) Analysis of variance for naupliar MLV indicated a significant (p=0.01) difference between control (2 ppb), intermediate (18-186 ppb), and highest (350 ppb) copper levels. Swimming speeds significantly increased at sublethal copper levels but rapidly declined at or near the lethal level (Figure 18-6.) The shift in distribution of linear velocities for test groups at these three exposure levels is clearly illustrated by frequency histograms (Figure 18-4).

In the third experiment, *B. improvisus* nauplii (from South Carolina) were exposed to Cu levels ranging from control (3 ppb) to 190 ppb for 24 hours at 25°C. Following video taping at 24 hours, nauplii were transferred to filtered seawater with algal diet and reared at 25°C. At 24 hours, post-exposure mortality at 190 ppb was 100 percent; at 150 ppb, 20 percent. Rearing to cyprid stage indicated no significant mortality differences between controls and nauplii exposed up to 98 ppb Cu. However, development time appeared delayed by Cu exposure as low as 50 ppb (Table 18-4).

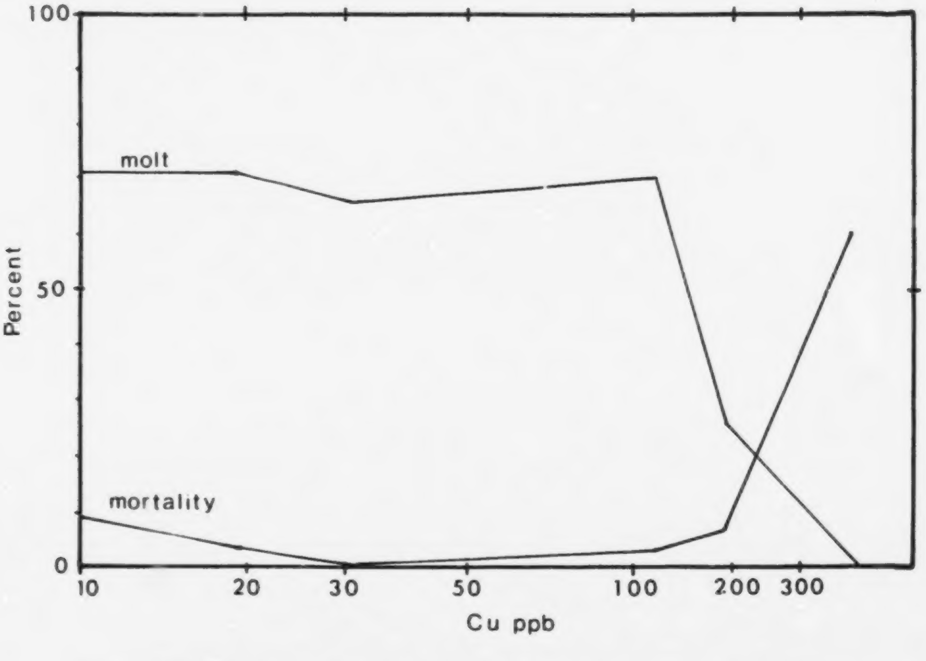
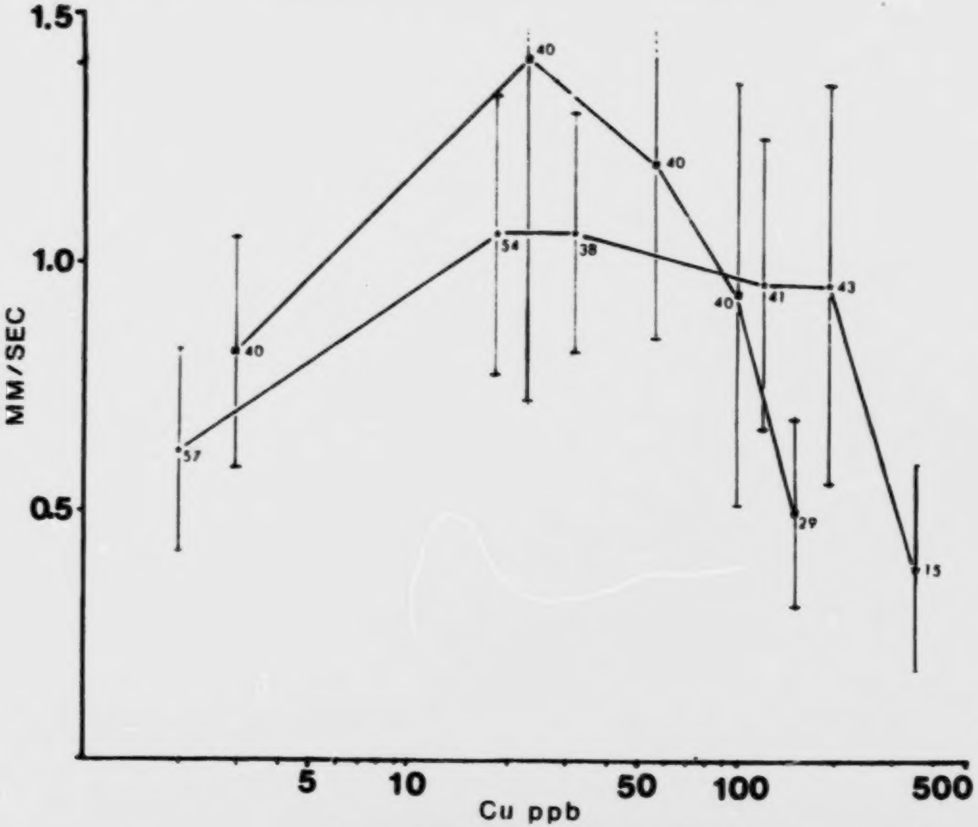


Figure 18-5. Mortality 24 Hours Post Exposure and Percent Larvae Molting to Stage III, 48 Hours Post Exposure, for Balanus amphitrite Stage II Nauplii Exposed to Various Copper Concentrations for 24 Hours at 20°C.



NOTE: Squares indicate *Balanus improvisus* nauplii at 26°C; stars indicate *Balanus amphitrite* nauplii at 20°C. Standard deviation bars and number of paths are indicated.

Figure 18-6. Mean linear velocity (mm/sec) of stage II barnacle nauplii following 24 hour exposure to different copper concentrations.

Table 18-4. Percent mortality and development time to cyprid of Balanus improvisus larvae following 24 hour exposure of stage II nauplii to different copper concentrations at 26°C.

Cu (ppb)	N	Mortality (%)	Days to Cyprid Range	Days to Cyprid Mean
3	75	19	7-15	10.2
25	81	25	7-15	10.7
47	59	19	8-16	11.7
77	64	19	9-16	12.6
98	77	25	9-14	11.6
150	76	61	8-15	10.3
190	70	100	–	–

NOTE: During the post-exposure period, larvae were reared on a mixed algal diet of *Tetraselmis suecia* and *Thalassiosira pseudonana* at 26 ± 1°C.

Swimming speeds of the above nauplii were significantly (p=0.01) increased at 25 and 50 ppb Cu relative to controls, nearly indentical at 98 ppb Cu, and significantly decreased at 150 ppb Cu (Figure 18-6). Although MLV of control and 98 ppb Cu populations were equal, the rate of change of direction (RCDI – absolute value of angular velocities in degrees/second) was substantially different (Table 18-5). These data show that the nauplii not only changed swimming speeds but also tended to change swimming patterns with increasing concentrations of Cu. Examples of path outlines and their computer assigned RCDI values are shown in Figure 18-7.

Table 18-5. Rate of Change of Direction (RCDI) and Mean Linear Velocities of Balanus Improvisus Stage II Nauplii after 24 Hour Exposure to Indicated Copper Levels at 26°C

Cu (ppb)	N	Linear Velocity (mm/sec)	RCDI (deg./sec)
3	40	0.82	130
24	40	1.43	140
57	40	1.23	151
98	40	0.83	166
151	29	0.49	188

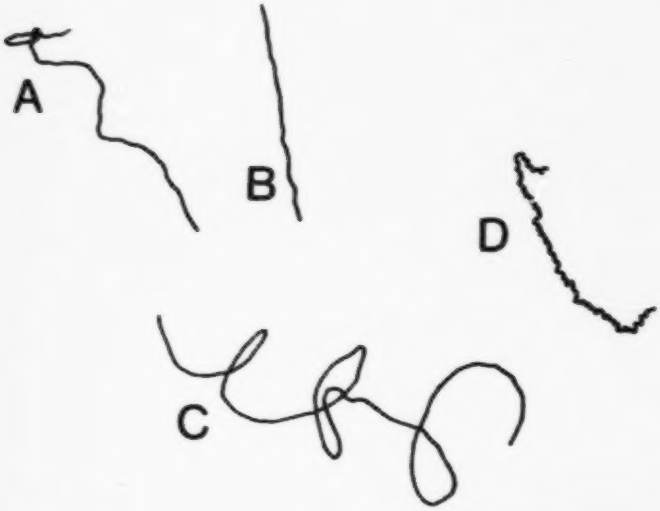


Figure 18-7. Examples of computer tracked paths for stage II Balanus improvisus nauplii and assigned rate of change of direction values (degrees/sec.): A) 114, B) 53, C) 152, D) 373.

NOTE: Paths A, B are typical of control conditions; path C occurs more frequently with copper present; path D was observed only above 50 ppb copper.

DISCUSSION

Initial results have demonstrated possible use of invertebrate larval swimming behavior as a sublethal response index. It has also been shown that for this index to be reliable, the effects of basic experimental variables such as temperature and light regime on the swimming response of test organisms should be understood.

Although previous studies on cirriped and brachyuran larvae (2, 11, 25) indicate no phototactic response is evident above 650 nm, cirriped nauplii appeared to exhibit a weak response to the present substage light. Spectral sensitivity of the species tested appears to extend further into the red than previously reported.

Balanus venustus and *B. amphitrite* nauplii exhibited two responses to sudden changes in light. The cessation of swimming by nauplii following a sudden light decrease is similar to the "sinking response" described for brachyuran larvae (10). The increase in swimming velocity of larvae following light increase cannot be fully described without consideration of possible directional response. Of practical relevance to our video taping and analysis procedures is that both responses are of brief duration, limited to the first three to five seconds of an acute illumination change, and can be detected from calculations of the MLV. The significance of these responses are not yet understood. Similar behavioral characteristics were not observed in stage II nauplii of *Chthamalus* and are also reported lacking in *Balanus balanoides* (6).

Temperature is known to directly affect swimming rate of invertebrate larvae (15, 23). All temperatures tested here on second stage nauplii were within ranges allowing complete development of the barnacles (14). It is clear for all species tested that small temperature shifts can alter swimming speeds. The basic influence of temperature observed on larval swimming rates is probably primarily a function of species and thermal history, yet initial results with these barnacle nauplii suggest other factors may prove significant. For example, *Balanus improvisus* collected from Rhode Island and South Carolina and maintained at identical laboratory conditions for over one month, released larvae having apparently different swimming rates relative to temperature. Maximum MLV occurred at 25°C for South Carolina larvae and at 22°C for Rhode Island larvae. Similar differences in response were observed in *B. amphitrite* from South Carolina and *C. fragilis* from Rhode Island. Replicate tests with different hatches are needed to confirm whether geographical variations persist.

To determine whether swimming patterns of barnacle nauplii are altered by toxic substances, stage II nauplii were exposed to different copper concentrations. Our exposure time to copper was limited to 24 hours. No algal food was added during this period to preclude complexing of the metal by the algae. Deprivation of food for 24 hours is not deleterious to the larvae. A 24 hour LC50 of between 200-350 ppb Cu for *B. amphitrite* nauplii at 20°C and between 150-200 ppb Cu for *B. improvisus* nauplii at 26°C was observed. Similar toxic concentrations have been reported for *Balanus crenatus* nauplii (19) and *Balanus eburneus* nauplii (5). Cyprid larvae or adults were more resistant to copper in both these studies.

Differences in LC50's observed for *B. amphitrite* and *B. improvisus* nauplii may be related to temperature. Higher temperatures can increase copper toxicity (3) or at least give this appearance in short-term experiments (21). Weiss (26), however, found *B. amphitrite* to be more tolerant of Cu than *B. improvisus* at settlement. In either case, toxic effects of Cu are often cumulative (3); both LC50 levels and sublethal effects probably occur at lower concentrations with increased exposure times.

For 24 hour exposures, concentrations of Cu below 100 ppb were clearly sublethal to the nauplii tested. Delay in development of *B. improvisus* nauplii occurred at 50 ppb Cu and changes in swimming behavior were evident at 15-25 ppb Cu. At the lowest Cu test levels, responses were restricted to increased MLV, but at higher sublethal concentrations MLV was depressed and swimming patterns became atypical. A stimulatory effect of very low levels of copper on swimming activity has also been reported for brook trout (9.5 ppb Cu) (9) and with sea urchin sperm (<20 ppb Cu) (28).

Forward and Costlow (12) also observed increased swimming activity of crab larvae exposed to 0.1 ppm of an insect juvenile hormone mimic, although larval development was not perceptively affected until 1.0 ppm was reached. On the other hand, sublethal concentrations of mercury and oil are reported to depress activity of marine crustaceans at nearly all levels tested (8, 18). Stebbing (23) suggests that apparent stimulatory effects of heavy metal ions on growth in marine hydriods and other groups are often only temporary and may represent a normal response to stressors.

Observations on swimming of *B. improvisus* nauplii indicate that not only the linear velocity, but also the pattern is altered by Cu. Nauplii swimming in convoluted paths (Figure 18-7) tends to increase in number in the presence of copper above control levels. As copper concentrations exceed 50 ppb, paths with a distinct "wobble" became evident (Figure 18-7). This latter pattern is possibly a consequence of impaired or abnormal beating of appendages. This would lead to reduced feeding abilities, as feeding in cirriped larvae is a direct function of appendage movement. The increased development time to cyprid observed at higher sublethal copper concentrations may be the result of difficulties in feeding.

The present study has consistently observed altered swimming behavior of cirriped larvae at Cu concentrations far below 24 hour toxic levels. Basic changes in swimming speed *per se* may prove useful indicators of pollution stress, but also of great interest are additional effects on larval motile responses to environmental stimuli or cues (light, chemical, gravity, etc.). The latter may prove more meaningful in predicting safe levels of pollutants. If short-term behavioral reaction can be satisfactorly correlated with long-term detrimental effects, the potential exists for rapid screening of toxic levels using this motile behavioral qualification technique. Further studies relating observed behavioral responses to other physiological parameters, and ultimately larval success, are planned.

ACKNOWLEDGEMENTS

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USE OF A LABORATORY PREDATOR-PREY TEST AS AN INDICATOR OF SUBLETHAL POLLUTANT STRESS

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ABSTRACT

A method is presented to quantify the effects of sublethal stress on newly hatched and older ichthyoplankton using predation vulnerability as a measurable parameter. A laboratory predator-prey system was developed and tested using sublethal thermal shock (10°C above ambient water temperature) as the stressing factor. *Fundulus majalis* was chosen as the predator and larvae of *Menidia menidia* and *Paralichthys dentatus* as prey organisms. Predation interactions were quantified by recording all attacks, escapes, and captures, allowing comparison of escape probabilities (no. escapes/ attack) for control and shocked prey groups.

Predator escape ability of four and six week old larvae *M. menidia* was significantly impaired following a 15 minute, +10°C thermal shock in summer (thermal test exposure = 30.0°C). Newly hatched and two week old shocked *M. menidia* were not significantly different from controls. Tests with *P. dentatus* showed an increase in total number of escapes following 10°C thermal shock in late fall tests (thermal test exposure = 25.2°C).

The potential for laboratory predator-prey tests as behavioral bioassays to assess sublethal pollutant stress is evaluated, with consideration given to the several techniques developed to date.

INTRODUCTION

The present study was undertaken to develop a laboratory predator-prey test system to evaluate relative ecological fitness of larval fish following a sublethal pollutant stress. Thermal shock was employed in this case. Behavioral bioassays are considered to be more sensitive indicators of low-level stress in comparison with mortality bioassays (22). Hence, behavioral tests should serve to identify the less conspicuous, but nonetheless important limiting effect that real-world sublethal stress can have on organisms. In the case of laboratory predator-prey tests, changes in prey escape success serve to indicate changes in ecological fitness, which can affect natural mortality rates in localized populations.

MATERIALS AND METHODS

For this study, larval prey species were restricted to those available from laboratory culture. Wild ichthyoplankton were not considered because of the potential for damage due to capture methods, and the difficulty in acquiring adequate numbers of a single species of the same age. The cultured larval prey species used were *Menidia menidia* and *Paralichthys dentatus*. Six hatched lots (∿ 500/lot) of *M. menidia* were reared to six weeks of age and tested during this period. Studies with *P. dentatus* were limited to newly hatched larve only, as this species experiences high mortality at time of first feeding. All larvae were reared at the prevailing Narragansett Bay water temperature (*M. menidia*, summer–20.5 ± 0.7°C; *P. dentatus*, late fall–15.1 ± 0.8°C).

An attempt was made to correlate prey and predator species. *Fundulus majalis*, a carnivorous near-shore predator, was chosen as a spatially coexisting predator of estuarine larval fish (6). Larvae of *P. dentatus* are not highly correlated with near-shore predators since they are usually found offshore at hatching. However, this species was utilized to provide a larval fish with different swimming abilities. *Paralichthys dentatus* relies on high fecundity for successful development and eventual recruitment. Larvae of this reproductive strategy are usually weak-swimming relative to larvae of a species such as *M. menidia*, which has a lower fecundity, but relies on advanced morphological development and strong swimming capabilities at hatching.

Biological variables controlled for this study include: reproductive condition of predator (a L:D 10:14 photoperiod was used to minimize reproductive development interference); nutritive condition (all predators were fed a mixed daily diet of Tetramarin flake food and adult frozen *Artemia salina* until 48 hours prior to a test); predator size in relation to prey size (preliminary tests indicated selection of a predator size of 6-8 cm total length (TL)); feeding periodicity (all tests were performed at the same time of day); and hunger state (all predators were starved 48 hours prior to a test).

Forty-eight hours prior to a test, each predator was placed into an experimental predation tank, which consisted of a polypropylene tub 30 cm diameter x 12.5 cm deep, with a clear Plexiglas bottom (Figure 19-1). Test tanks received a continuous flow of filtered seawater (∿ 400 ml/min.) pumped directly from Narragansett Bay. All predation tests occurred between 1300 and 1500 hours at ambient bay water temperatures.

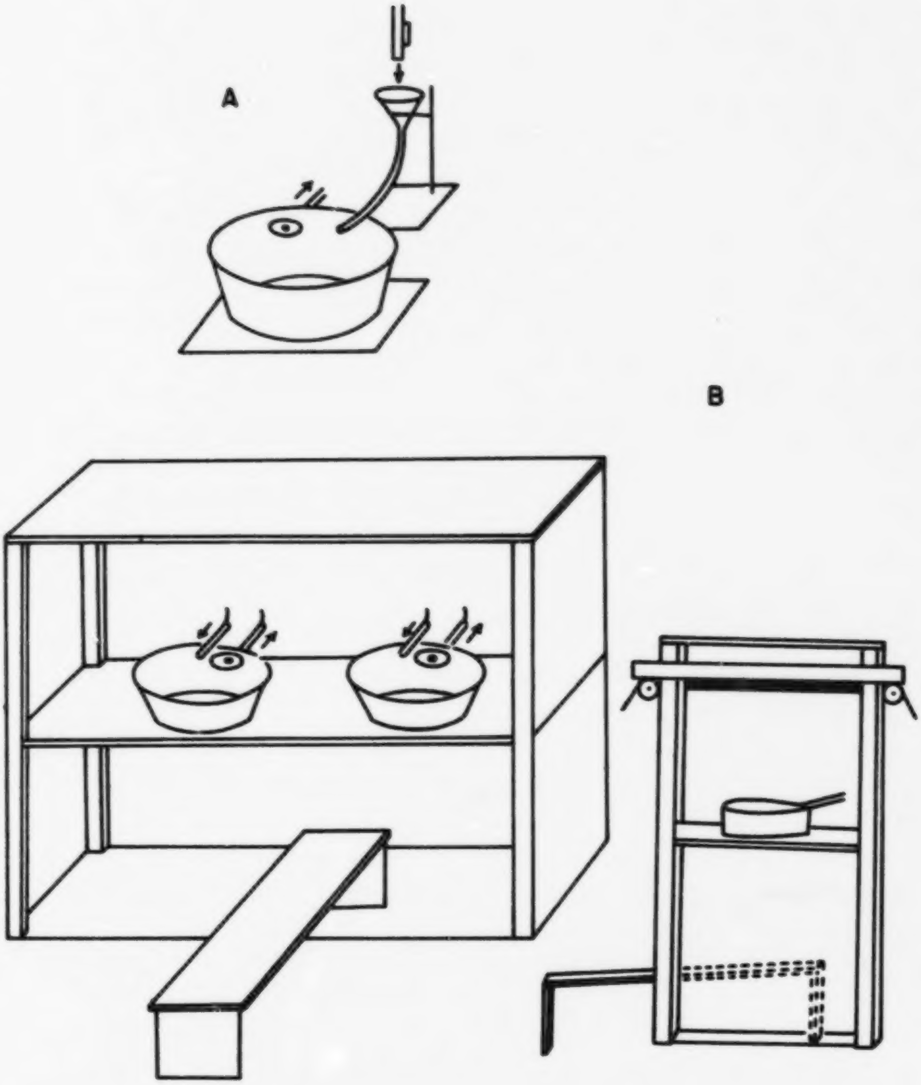

Figure 19-1. Predation tank and inflow funnel (A) and (B) Observation Bench, front and side view (normally covered with black plastic).

Thermal Shock Procedures

Larval *Menidia menidia* were transferred in 100 ml polypropylene beakers with a horizontal slit 1 cm x 2 cm wide cut 1 cm above the bottom, and covered with 240 u nylon screening. Larvae were placed into the beakers in groups of 10, four hours prior to a test and maintained at ambient water temperature (20.5 ± 0.7°C). For the shock tests, a seawater bath was preheated to 30.0 ± 0.6°C (△ T = 9.7 ± 0.7°C) and beakers were placed into the heated water at six minute intervals. Containers were aerated throughout the shock procedure. As a 15 minute exposure period was completed, a beaker was immersed in seawater 1°C above ambient for a five minute cooling period. The larvae were introduced into predation tanks via a funnel filled with incoming seawater (Figure 19-1). Larvae were added at six minute intervals, and most larvae were eaten by the predator within the first three minutes following introduction. All control larvae were treated in the same manner as shocked larvae, but with transfer containers held at ambient water temperature rather than a higher temperature.

Larvae of *Paralichthys dentatus* are prone to damage in screened beakers because of weak swimming ability and great sensitivity to handling (Grace MacPhee, personal communication). Therefore, intact 100 ml polypropylene beakers were used as transfer vessels. Ambient bay water temperature during these tests was 15.1 ± 0.8°C. Groups of 10 larvae were shocked by gently pouring the contents of each 100 ml beaker into a glass culture bowl (12.5 cm dia.) containing 100 ml of seawater preheated to 25.2 ± 0.8°C (△T = 10.1 ± 0.6°C). After the 15 minute exposure period, larvae were siphoned into the predation tank using silicon tubing (9.5 mm dia.). Introductions of larvae to the thermal treatment were again staggered at six minute intervals, as with *M. menidia*. Control larvae were treated in the same manner as shocked larvae, but with transfer to 100 ml of seawater at ambient water temperature.

Quantifying Predator-Prey Interactions

During the predation tests, all attacks, captures, and escapes were observed from below and recorded using an Esterline-Angus event recorder. The best visual field for recording observations was achieved by placing two opposing light sources (two fluorescent bulbs) above, yet just outside of the visual range of an observer directly below the tanks, and placing a flat black background over the tanks (Figure 19-1). This system permitted accurate recording of predator-prey interactions involving organisms as small as four mm. Significance of changes in escape probabilities, expressed as no. escapes/attack, were tested using the Wilcoxon distribution-free rank sum test (13).

RESULTS

Results of predation tests for *Menidia menidia* are grouped by prey age categories (Table 19-1). The two oldest larval groups of *M. menidia* (four week and six week old) experienced a significant decrease ($P < .01$) in the number of attacks, escapes and escapes/attack for shock tests relative to control groups. The two youngest age groups of this species (newly hatched and two week old) did not show significant treatment differences in any of the parameters measured.

Larval *Paralichthys dentatus* of only one size class (4 mm TL) were tested. The results stand in contrast to the findings with older *Menidia* larvae. A significant increase in escape ability ($P < .01$) occurred in *P. dentatus* larvae following thermal shock (Table 19-1).

DISCUSSION

Relationship of Results to Upper Thermal Limits

Results of tests with four and six week old *M. menidia* indicate a possible adverse effect following an acute thermal increase to 30.0°C from an acclimation temperature of 20.5°C. The magnitude of this thermal elevation is close to the one hour TLM value of 31.4°C given by Hoff and Westman (14) for juvenile *M. menidia* acclimated to 20°C. The present study points to the increased sensitivity of behavioral stress indices to monitor effects of short-term or low level pollutant stress. Indeed, these findings strongly contrast the view of Austin *et al* (1). Based on mortality studies of a 13 minute shock of 14°C above a 20°C acclimation temperature, he concluded that this treatment would not have any important effects on survival of larvae of this species.

The absence of significant differences in escapes in newly hatched and two week old stressed *M. menidia* vs. the controls may be real, or could be due to the low number of tests run and the high variability observed within the shock groups. More data are necessary before final conclusions can be made on the sensitivity of these younger larvae to thermal shock.

The increase in escape probability following thermal shock with larvae of *P. dentatus* may be due to an increase in altertness or in frequency of locomotory movements. Because the ambient water temperature was lower in tests with *P. dentatus* (15.1°C), the thermal shock did not approach reported lethal levels (32.0°C CTM at 15°C acclimation, Hoss *et al* (25)). Increased escape ability has been reported by Coutant (8) in juvenile *Salmo gairdneri* when thermal shock temperatures are well below lethal levels.

Potential Mechanisms of Thermal Shock Effects on Predator Avoidance

Although the phenomenon of differential predation in thermally shocked fish is now well documented (8, 25, 27), causal mechanisms for changes in vulnerability following thermal shock are not known. It has been demonstrated that the central nervous system is highly sensitive to temperature fluctuations (4, 23). The thermal sensory receptors are believed to consist of cutaneous free nerve endings (3), yet behavioral response to thermal shock is not necessarily limited to free nerve endings. Blood chemistry, membrane permeability, and

Table 19-1. Results of Predation Tests: Pooled Data for Each Age Category (Mean and 1 S.D.)

Group	Mean # Attacks		Mean # Escapes		Tot. # Escapes / Tot. # Attacks		Total # Introduced	
	S	C	S	C	S	C	S	C
M. menidia								
1-3 day; 4mm	64.8	63.0	17.3	17.0	0.268	0.270	150	150
N = 3 S; 3 C	(11.0)	(7.6)	(12.0)	(4.6)	(0.139)	(0.043)		
2 week; 9.8 mm	53.0	61.4	26.0	33.0	0.491	0.537	120	150
N = 4 S; 5 C	(19.5)	(9.7)	(16.5)	(8.6)	(0.131)	(0.056)		
4 week; 12.2 mm	60.4**	74.3	32.4**	45.6	0.536**	0.613	600	420
N = 20 S; 14 C	(13.1)	(13.1)	(12.1)	(12.9)	(0.089)	(0.067)		
6 week; 23.5 mm	25.8**	35.5	15.3**	25.4	0.592**	0.715	160	160
N = 8 S; 8 C	(7.1)	(8.9)	(6.3)	(9.3)	(0.097)	(0.089)		
P. dentatus								
1-3 day; 4 mm	66.0*	56.8	17.8**	8.4	0.270**	0.148	240	249
N - 5 S; 5 C	(8.6)	(2.9)	(6.3)	(2.5)	(0.061)	(0.038)		

NOTE: S = Shocked Group (15 min. duration, *M. menidia*
$\Delta T = 9.7^{\circ}C$, *P. dentatus*
$\Delta T = 10.1^{\circ}C$), C = Control Group. $^{*}P < .05$. $^{**}P < .01$.

other physiological effects have all been demonstrated to occur following thermal shock, and may cause behavioral changes (23). Laudien (18) and Murray (21) both note that the lateral line system in fish is highly sensitive to rapid temperature changes. It is possible that disruption of normal lateral line function may potentially decrease response to a predator's attack. Blaxter (7) considers the free neuromast system in larvae to play an important role in avoiding predation.

There is evidence that the lateral line may indeed be disrupted by a thermal shock. Dijgraaf (10) demonstrated that the spontaneous discharge frequency varies with temperature in the lateral line of *Xenopus* (Amphibia). Murray (20), also working with *Xenopus*, noted that a sudden temperature increase of $10^{\circ}C$ would decrease or even completely inhibit the spontaneous discharge frequency, followed by compensation back to normal levels. Sudden cooling would cause a sudden increase in frequency. If free neuromast and developed lateral line receptors of fish larvae react similarly to those of *Xenopus* following thermal shock, there are two periods when the normal receptor frequency would be altered and signal information from the system possibly masked or inhibited. The first would occur upon contact with water of increased temperature. Thus, upon interception with a thermal discharge, and if the temperature differential is high enough, complete inhibition may occur, cutting off all signals from the lateral line momentarily. Inhibition of spontaneous discharge probably does not pertain to the present study, since predators are absent during the initial 15-minute thermal shock period. However, this initial neural inhibition could render larvae which pass through a thermal discharge plume more vulnerable to predation. Next, following such a thermal shock, the larvae experience rapid cooling, which could result in a sudden increase in lateral line discharge frequency and possible distortion or masking of near-field environmental stimuli. This latter effect may be involved in the present study since cooling of larvae occurs just prior to the predation interaction.

Evaluation of Laboratory Predator-Prey Tests as Sub-lethal Indicators

Laboratory predator-prey tests, such as the one described here, can be valuable as a means of observing subtle, but ecologically significant effects of low pollutant levels. In developing such tests, it is important to evaluate the strengths and limitations inherent in laboratory techniques utilized by other investigators (2, 8, 11, 12, 16, 25, 27). One must consider which primary predation factors are being measured by each method. Bams (2) states that a differential predation situation is determined by three primary factors: discovery rate of the prey by the predator; attack rate on the prey; and capture rate of the prey.

Discovery rate is assumed to be approximately equal in all methods cited here since exposure of all prey groups to the predators is complete and equal. This parameter is best measured by recording the reactive distance to the prey item (Beukema, 5), a difficult task which is not addressed in any of the above techniques.

Attack rate can influence differential predation rate in that certain characteristics of the prey may be perceived by the predator and produce active selection. This behavior could occur in predation tests where simultaneous introduction of treated and control prey groups occur, as in the methods employed by Bams (2), Coutant (8), and Kania and O'Hara (16). However, as Bams noted, this parameter cannot be quantified by these methods because group identity of individual prey is not discernible during attacks.

Differences in capture rate between prey groups are a result of differences in prey ability to evade an attacking predator. The techniques used by Bams (2), Coutant (8), and Kania and O'Hara (16) cannot discern between differences in capture rate and differences in attack rate since the overall result of predation is measured, and not individual attacks and captures. It is in this regard that the method devised by Yocum and Edsall (27) is superior, because it specifically measures the actual instantaneous predation rate as affected by changes in the rate of capture. Although Sylvester (25) also measured rate of capture (in terms of mean survival time), Yocum and Edsall found excessive variance between predator groups in time-to-capture when using his method. Of the various techniques, those of Yocum and Edsall (27), Bams (2) and Coutant (8) are considered best suited for laboratory predator-prey studies. The technique used by Kania and O'Hara (16), is similar to that of Coutant and Bams, but with the addition of an escape area. This modification limits its application to those prey species with a specialized behavioral characteristic of shallow water refuge. Also, there is a probable complication of learning, as prey become familiar with the predator's area, and the "safe", shallow, screened area used in the tests. Finally, the 60 hour test duration is fairly lengthy.

In this particular study with larval fish prey, Bams' (and Coutant's) method of simultaneous presentation of prey from different treatment groups could not be utilized, as a tag to distinguish prey treatment groups is necessary. Common methods for identification, such as fin clipping and cauterization branding, were not feasible with larval prey. A visual dye is unacceptable because of potential alteration to predator-prey relationships due to conspicuousness of prey and color preferences in the predator. A number of fluorescent dyes were tested, but successful dyes were found to alter normal behavior in fish larvae (*Pseudopleuronectes americanus*). Efforts to label fish larvae with a radioisotope were also unsuccessful. Due to these difficulties

Yocum and Edsall's technique of recording individual attacks, captures, and escapes was adopted.

Both methods have intrinsic advantages and disadvantages. Bams' method allows groups of predators to select between treated and untreated prey simultaneously. However, the test statistic used by Bams is a biased estimate of instantaneous mortality rate (2, 8). The method used by Yocum and Edsall records the actual instantaneous mortality rate in terms of attacks, escapes, and captures. These parameters allow a more accurate representation of changes in escape capabilities. In this latter method, prey treatment groups are separated, and the predator does not simultaneously compare prey groups. In a thermal plume area, where predators have been observed to attack thermally-shocked prey (8), it is not likely that shocked and unshocked prey would be in close proximity to one another. However, in studying the effects of other pollutants, simultaneous comparison of prey behavior may be an important factor in differential predation, and should be considered.

A number of biological variables which should be considered in designing a laboratory predator-prey test system, including coexistence of predator and prey in nature (spatial and seasonal); plausible prey-predator relationship; size relation prey-predator; reproductive condition of predator; nutritive condition of predator and prey; feeding periodicity of predator; and hunger state of predator. Control of many of these variables has already been described in the Methods section. Perhaps one of the most difficult to control is satiation (hunger state) of the predator. Satiation state may affect prey risk (5). If a predator is less motivated to eat, attack efficiency may not be as high, thus artificially increasing escape rate of prey as measured by Yocum and Edsall's method. Prey size will affect time to satiation in a predator, and must, therefore, also be controlled. Optimal prey size can be estimated by calculating a prey thickness to predator mouth size ratio with 0.5 as optimal (17, 26). However, even with an optimal prey size and a set deprivation schedule, individual variability is often substantial. Procedures to categorize motivation state of the predator are recommended for laboratory predator-prey tests in order to eliminate this variability. In the present study, the total mean number of larvae captured per test was calculated for all tests within each prey age group. A minimum percentage of this mean was chosen as an indicator of adequate feeding motivation. A 75 percent limit described a minimum level of 22 larvae captured in tests with four week old *M. menidia*. Because six week old larvae were larger, the capture minimum was narrowed to 80% of the mean total larvae captured, giving a lower limit of eight larvae. All tests in which this minimum capture level was not reached were excluded from statistical treatment of data. In tests with larvae younger than four weeks, predators did not reach satiation before completion of the test, and establishment of a minimum capture level was unnecessary.

Laboratory predator-prey testing techniques should prove to be a useful tool in future pollution research. As noted, the various techniques available offer different approaches to the question of changes in prey vulnerability. The relative merits of each must be weighed with due consideration to the normal ecology of the predator and prey utilized, and the biological variables which must be controlled.

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BURROWING ACTIVITIES AND SEDIMENT IMPACT OF *NEPHTYS INCISA*

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ABSTRACT

It is suggested here that benthic deposit feeders are an important faunal group contributing to the flux of materials, including pollutants, between the benthos and overlying water. The present study has documented the burrowing and feeding activities of one dominant deposit feeder, the polychaete worm. *Nephtys incisa*, at a series of test temperatures spanning the annual thermal range (0-24°C) of Narragansett Bay, R.I. New burrow development and feeding are coupled events as the worm penetrates and ingests sediment. Each new burrow is usually continuous with recently abandoned burrows, which results in extensive perforation of the benthic sediment. Then as *Nephtys* ventilates its burrow for respiratory purposes, sediment oxygenation along the entire subsurface burrow network also occurs. Rate of new burrow building ranges from one/20 days at 0°C to one/day at 24°C.

It is hypothesized that *Nephtys* burrowing, feeding and irrigation activity contributes significantly to substrate conditioning for development of the aerobic benthic compartment. Doubtless, pollutant diagenesis is also directly influenced by this creation of an oxidative environment, resulting in significant pollutant fluxes to and from the benthos.

INTRODUCTION

The polychaete worm, *Nephtys incisa*, is common in silty-clay sediments of the northern Atlantic estuarine and coastal waters. Its dominance in fine sediment is a unique departure from other *Nephtys* species, all reported to be active carnivores inhabiting poor to well-sorted sands (Clark, 1962; Clark *et al*, 1962). To better understand the anomalous, silty-clay habitat preference of *N. incisa*, information regarding its in-sediment activities was pursued, primarily through the use of laboratory microcosms.

Soft-bodied organisms that burrow into the sediment generally do so for predator avoidance, at the minimum. Those which burrow continuously through the sediment, such as the Nephteidae, Nuclionid bivalves and Haustorid amphipods, do so- to obtain food either as predators or deposit-feeders. This vagile or wandering mode of life requires adaptations, not only for burrowing, but also for obtaining sufficient food and dissolved oxygen in this environment. The specific questions that have been investigated concern adaptations for burrowing, feeding and irrigation in *N. incisa*. The present paper will address certain questions of burrowing activities, while the two subsequent papers (Davis, 1979 b,c) will deal with feeding and irrigation activities in *N. incisa*. These activities are interrelated in that continuous sediment burrowing is generally a feeding adaptation and necessitates further adaptation to obtain well-aerated seawater while moving through the sediment.

N. incisa occurs in estuarine, shallow coastal waters and across the Atlantic continental shelf from Chesapeake Bay northward to Nova Scotia, Greenland and Iceland, and along the European coast from the North Sea, the Baltic Sea, and south into the Mediterranean (Pettibone, 1963; Thorson, 1946; Bellan, 1969). Reported population densities include 600/m^2 in Long Island Sound (Sanders, 1956), 300-600/m^2 in Narragansett Bay (Davis, Phelps and Morrison, unpublished), and up to 1500/m^2 in Buzzards Bay (Sanders, 1960). Population age structure has been examined temporally by Sanders (personal communication) who has observed three and sometimes four year classes, with each new year class appearing as 2 mm worms during early spring. Density of *N. incisa* can be correlated with sediment clay-silt content (Sanders, 1956), pollution gradients (Farrington, Quinn and Davis, 1973), and possibly meiofauna density (Tenore *et al*, 1977).

The types of burrows found among infaunal polychaetes range from totally permanent to highly temporary. In the case of completely vagile worms such as the Nephteidae, Nereidae and Glyceridae, this in-sediment wandering may lead to burrow galleries and multiple openings to the surface. This mode of burrowing is generally adapted to exploit debris or prey on the sediment surface while minimizing exposure to predators. Two or more openings to the water also permit efficient one-way irrigation to obtain dissolved oxygen. *Glycera alba* produces such a gallery, using various burrow openings as prey vibration conduits and will even intercept moving prey at other gallery openings (Ockelman and Vahl, 1970). *Nereis diversicolor* has a similar gallery to better exploit debris on the sediment surface. An interesting adaptation for secondary filter feeding in this species was described by Harley (1950). Under certain conditions the respiratory irrigation stream is directed into a mucous funnel which is then eaten. Other vagile polychaetes burrow to exploit subsurface organic-containing sediments, for example the Capitellidae, Maldanidae and Paraonidae. The capitellid *Heteromastus filiformis* develops semipermanent vertical burrows to reach deeper sediment in which it continually burrows and deposit-feeds. Oxygen exchange occurs near the sediment-water interface using modified posterior segments when the worm surfaces to defecate (Linke, 1939). *Paraonis* spp. continually burrow to form a deposit-feeding ring within a single stratum of high organic content (Gripp, 1927, cited in Schafer, 1962). When the concentric burrowing reaches 8-10 cm in diameter, the worm burrows to a new stratum to begin another feeding ring.

Partially or non-vagile families such as the Arenicolidae and Chaetopteridae possess U-shaped burrows and irrigate for the dual purposes of feeding and respiratory exchange. Families with the least sediment contact, termed tubicolous polychaetes, include the Sabellidae, Onuphidae and Serpulidae. These worms develop permanent tubes lined with mucopolysaccharides, shell debris, sand grains or calcite. They generally feed and ventilate above the sediment-water interface. There are many exceptions; for instance, the tubicolous Pectinariidae drag their sand grain tube horizontally through the sediment using it as an irrigation tube to the surface as they deposit-feed below.

The present investigation provides information on the microhabitat of *N. incisa* by describing the nature of its burrow and examining some of the variables influencing burrowing. Employing laboratory *in situ* microcosms, coupled with direct observation in the field for verification, this investigation has addressed such topics as the form and make-up of the *N. incisa* burrow, how it is constructed, what is its horizontal and vertical extent, and how the rate of burrowing is influenced seasonally as the worm is exposed to temperatures which can range from 0-24°C.

MATERIALS AND METHODS

Nephtys incisa used in this study were collected from a station north of Conanicut Island, Narragansett Bay, Rhode Island (Figure 20-1). This benthic station is characteristic of a large portion of the Narragansett Bay, where a clayey-silt sediment covers 60-75 percent of the Bay bottom (McMasters, 1960). Previous studies (Davis, *et al*., unpublished) found *N. incisa* density to drop off rapidly in the sandy sediment toward the Bay mouth and decrease gradually toward the northern head of the estuary, the latter possibly due to a pollution gradient (Farrington, *et al*., 1973).

N. incisa were collected by gently sieving Smith-McIntire grab samples and by SCUBA diver-collected box cores, which were then transported intact to the laboratory and held in flowing seawater. When temperature change was required, it was shifted at a rate of 2°C per week, which is comparable to the rate of temperature change in the field (Figure 20-2).

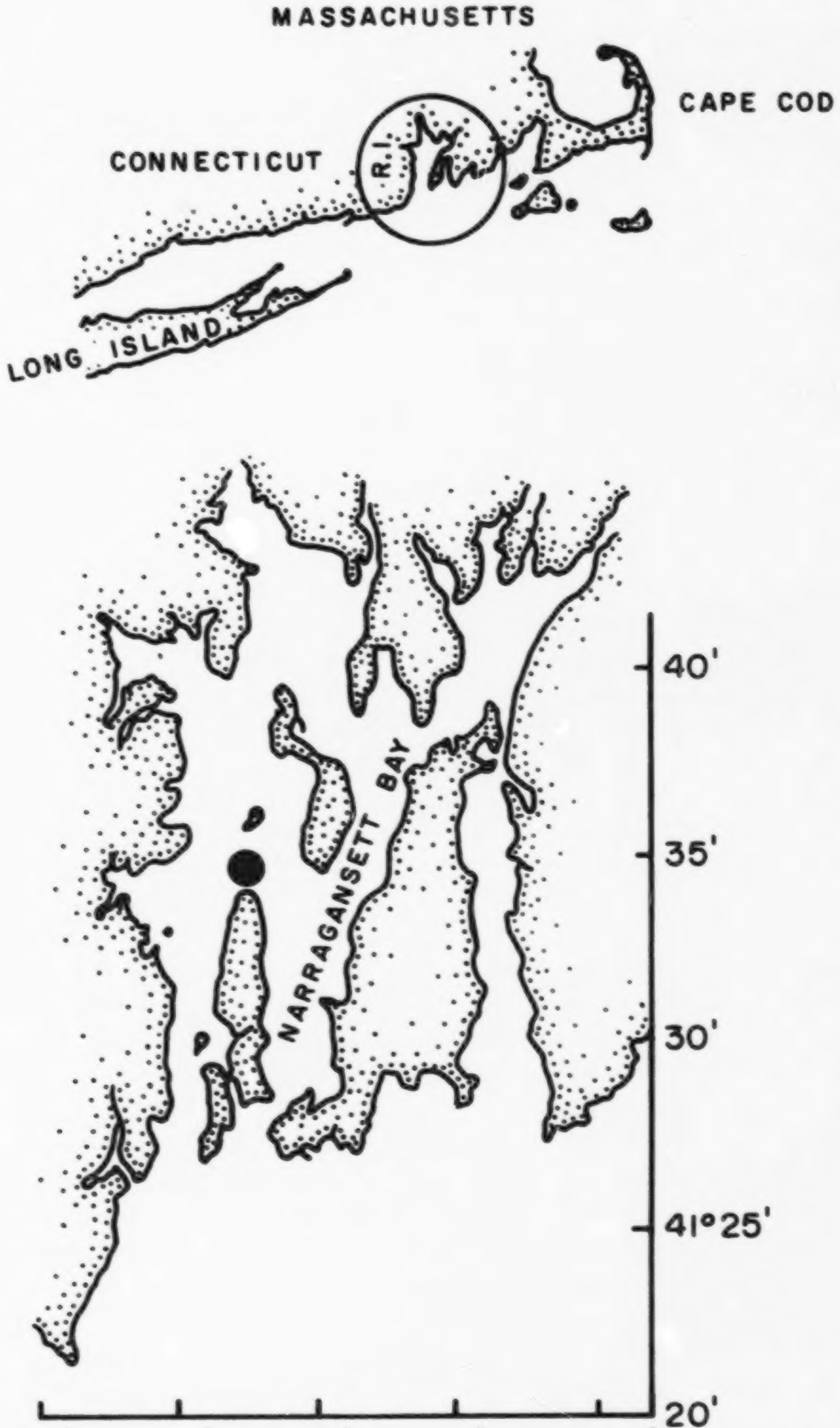
MASSACHUSETTS
CAPE COD
CONNECTICUT
R.I.
LONG ISLAND
NARRAGANSETT BAY
40'
35'
30'
41°25'
20'
30'
25'
20'
15'
71°10'

Figure 20-1. Narragansett Bay, Rhode Island, Collection Site.

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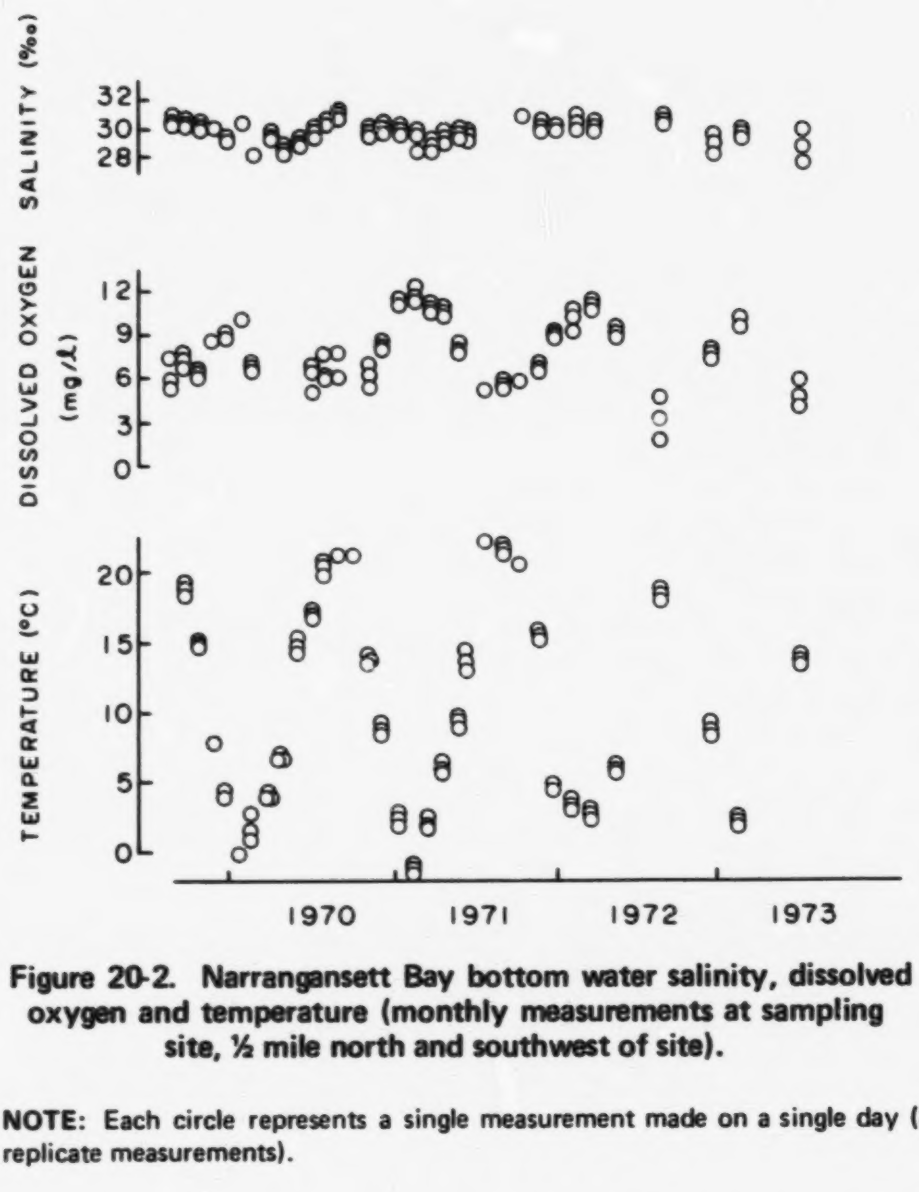

Figure 20-2. Narragansett Bay bottom water salinity, dissolved oxygen and temperature (monthly measurements at sampling site, ½ mile north and southwest of site).

NOTE: Each circle represents a single measurement made on a single day (3 replicate measurements).

Burrowing activities were described and quantified by observing single worms in sediment-filled thin aquaria (2 cm thick, 14 cm wide and 15 cm deep). These aquaria were maintained in flow-through systems at appropriate temperatures. The thin aquaria allowed free three-dimensional movement (worms can easily turn in 1 cm thick aquaria), yet also permitted direct observation of some activities with a stereomicroscope when the worms burrowed along the glass. The glass walls are considered to represent an obstacle to the worm not unlike buried rocks or bivalve shells, which are commonly observed in the field. Experimental worm density was one per experimental aquarium or one worm/28 cm^2. The aquaria were kept in the dark except for a two-minute interval once weekly when both sides of the squaria were photographed.

Patterns and rates of burrowing and sediment aeration were described from sequential photographs of the worm burrows constructed against the glass wall of the sediment-filled thin aquarium. Sediment aeration by local burrowing and irrigation was indicated by a change in sediment color from dark to light and provided a record of the worm's present or past location. This method relies on oxygen-sediment-color relationships proposed by Fenchel and Riedl (1970), Hayes (1964), Teal and Kanwisher (1961), Rhoads, Aller and Goldhaber (1977), and Aller and Yingst (1978). The absence of oxygen generally leads to a dominance of reduction reactions (eH<0) including formation of iron sulfides which blacken the sediment. At the point of change from dark to light color in the sediment, values for both eH (volts) and dissolved oxygen (mg/1) begin to increase from 0.0. Presence of oxygen is key to substrate oxidation reactions (eH<0). By quantifying the development of this color discontinuity against the thin aquarium glass wall where worms are burrowing, it is possible to document three parameters of burrowing activity: 1) the spatial-temporal extent of burrowing, 2) the effective new surface area of the sediment-water interface, and 3) the extent of sediment aeration. This record, visible against the aquarium wall, can be photographed at appropriate time intervals and activity quantified by counting burrows, measuring the surface area of burrow linings and by planimetry, measuring the volume of aerated sediment. Horizontal burrowing patterns were also described by recording temporal and spatial appearance of new burrow openings at the sediment surface in large sediment-filled dishes (single .2-.3 g worms in 3 x 6 x 4" deep sediment trays).

RESULTS

Description of the Burrow

Nephtys incisa actively penetrates fine sediments and establishes an open-ended burrow with no visible modification of the burrow wall except packing. It is not known if mucous, exuded onto parapodial setae during feeding (Davis, 1979b), is present in the burrow wall. The burrow is often W-shaped, but many variations exist. Back and forth motion of the worm with packing of the burrow wall by setal bundles creates a section which is closely fitted to the front and mid section of the worm. This precise fit permits flow-through irrigation by the parapodial cilia (Davis, 1979c). The occupied burrow is often continuous with a recently abandoned burrow posteriorly, which then continues to receive oxygenated water before it returns to the surface. Abandoned burrow segments gradually fill with suspended particulates and/or from the collapse of old burrow walls. There is also avalanching of surface floc into efferent and afferent burrow openings. The volume of suspended sediment transported in this manner into the deeper benthos then is proportional to total burrow volume.

Method of Burrow Building

New burrow building is accomplished as *N. incisa* first penetrates the wall of its existing burrow with an undulating proboscis, displacing small amounts of sediment. Worm position for this initial step is maintained through hydrostatic enlargement of the posterior segments. The worm next penetrates the sediment by lengthening the anterior segments and finally, as the head penetration reaches its forward limit, the pharynx everts, creating a bulbous cavity in the sediment. This type of sediment penetration has been described as "bolting" by Schafer (1962). He states that this is a common form of sediment penetration and is accomplished by contracting all body musculature. The resulting pressure forces coelomic fluid into the anterior region, forcing those segments without longitudinal muscles to expand and finally everting the pharynx at peak pressure. When the pharynx is re-inverted, *N. incisa* swallows a slurry of sediment which was created as the compacted sediment was penetrated. The whole sequence is repeated until the worm occupies the new burrow fully and has established a new opening to the surface. The entire process can usually be accomplished in less than an hour.

Spatial Extent of Burrowing

A series of observations were made to determine if burrowing followed some functional pattern *vs* random burrowing and also to determine the horizontal and vertical scope of burrowing. A typical sequence of new burrow formation is illustrated in Figure 20-3. This figure is a two-dimensional reconstruction of a three-dimensional activity which is typically only partially visible against the glass wall. It represents an example of burrowing but does not indicate a predictable pattern of burrowing. Figure 20-3 shows actual tracings from weekly photographs of a different burrow building sequence (a single worm over a six-week period at 18°C). Observations were also made with worms in large dishes of sediment so that horizontal movement could also be assessed without wall interference. Each new afferent burrow opening was mapped, with new openings connected as a series of vectors (Figure 20-4). At the time of the last recording, the worm is probably lying in a burrow between opening 10 and 11, with the head located toward opening 11. The magnitude of each horizontal vector was found often correlating with the size of the worm (Figure 20-5), with burrow length approximately 2-3 times the length of *N. incisa*. Yet exceptions do occur, as shown in Figure 20-4 by the short distance to afferent openings 2 and 4. By observing 30 six-week sequences of

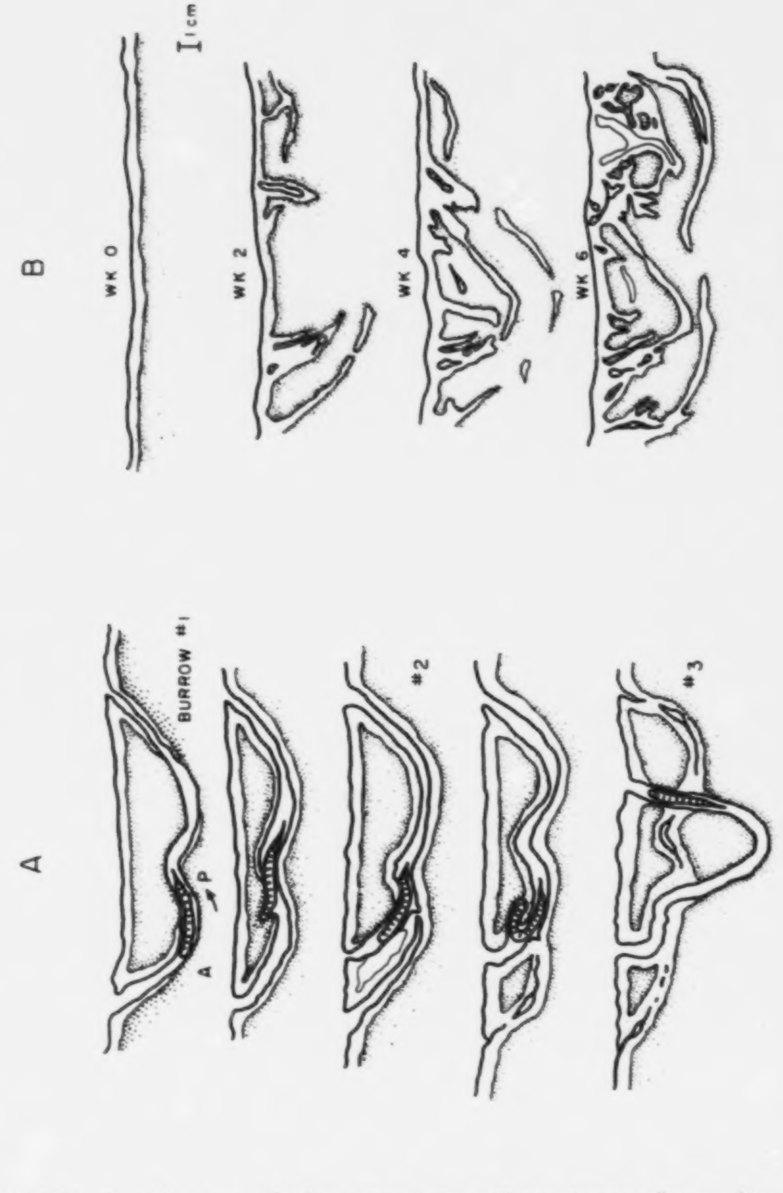

Figure 20-3. Vertical patterns of *Nephtys incisa* as seen against glass walls of a thin, *sediment-filled* aquarium.

NOTE: A. Diagram of the reconstructed sequence of new burrow building (burrows nos. 2 and 3) by a single worm, with collapse of original burrow (No. 1). "A", anterior, "P", posterior; direction of movement. B. Diagram of the vertical burrow network developed by a single worm and its persistence over time (18°C).

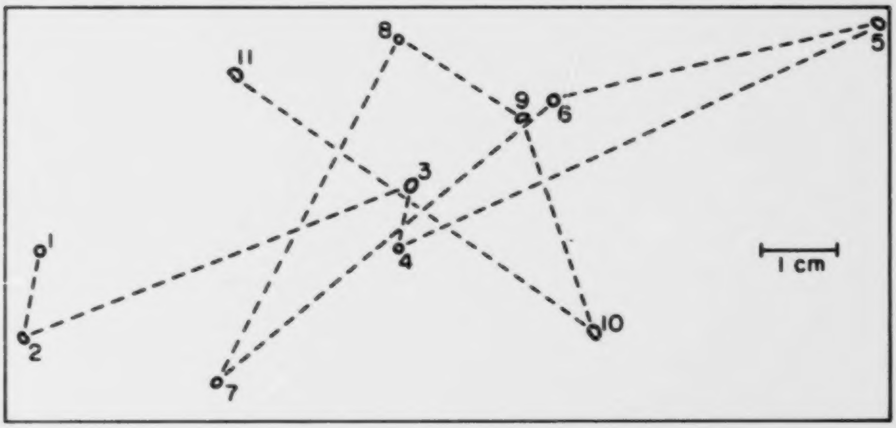

Figure 20-4. Horizontal burrowing pattern of a single Nephtys incisa as indicated by the sequence of new afferent burrow openings appearing on surface of large sediment-filled box over a period of 2 weeks (18°C).

NOTE: Afferent opening no. 11 indicates most recent burrow opening.

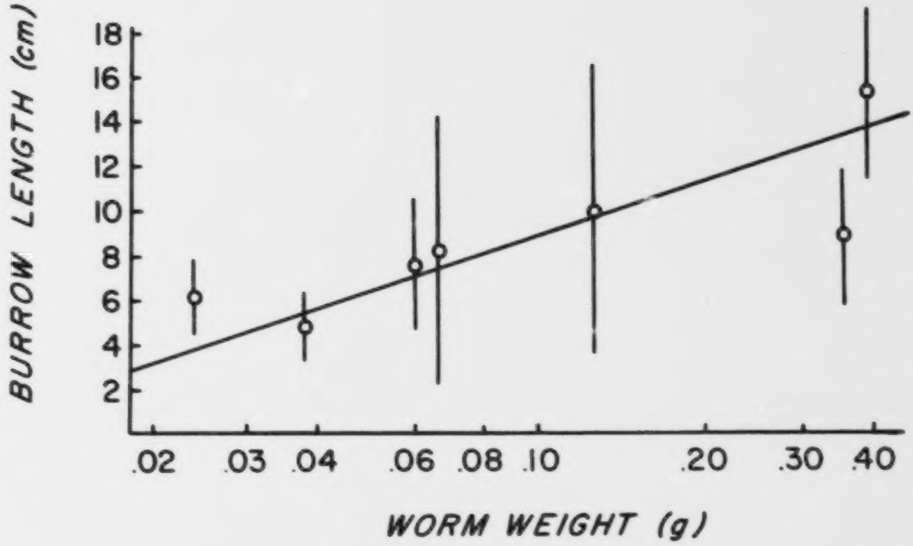

Figure 20-5. Relationships of size of N. incisa to burrow length using a flexible cm rule.

NOTE: Each vertical bar represents the range of 4-7 observations of a single worm of the indicated weight. N = 7 worms.

vertical and horizontal burrowing, it was concluded that no pattern of burrow gallery formation existed but that burrowing was a meandering extension of past burrows.

Depth of sediment penetration likewise correlates with worm size (Figure 20-6). The lower vertical limit of burrows for first-year worms (up to 0.1 g wet weight) is 2-3 cm beneath the sediment-water interface. Second-year worms (up to 0.4 g) limit burrowing to 7-8 cm. Three-year old worms (up to 1 g) may burrow as deep as 14-15 cm. *N. incisa* of all sizes may be found near the

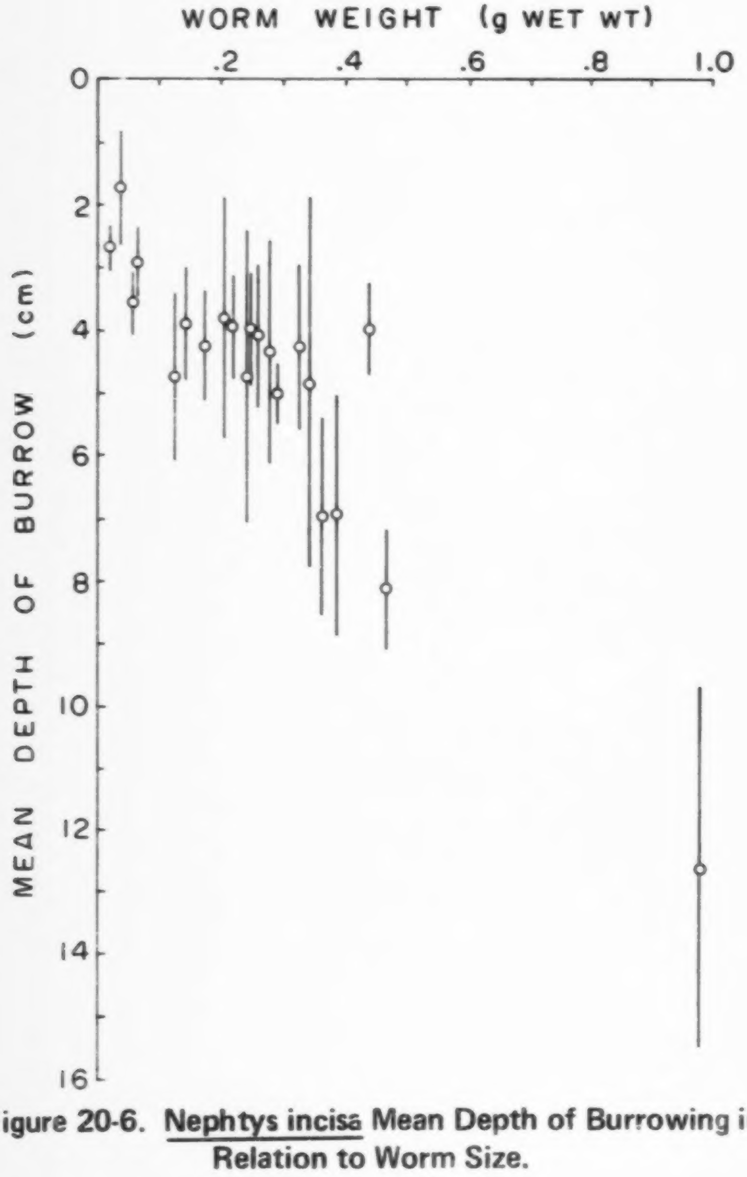

Figure 20-6. Nephtys incisa Mean Depth of Burrowing in Relation to Worm Size.

sediment-water interface, indicating that *N. incisa* may perforate sediment at all levels down to its size-limited depth.

Temporal Aspects of Burrowing

Rate of new burrow formation is temperature-dependent. Figure 20-7 illustrates both the numbers of new burrows formed per week and its reciprocal, duration of burrow occupancy, at five temperatures spanning the annual thermal range. At 0°C, burrow turnover averages one new burrow built every two weeks. At 6°, an average of 1.5 burrows are built per week; 3-3.5 at 12°; 3.5-4 at 18° and 6.5-7 burrows were observed per week at 24°C.

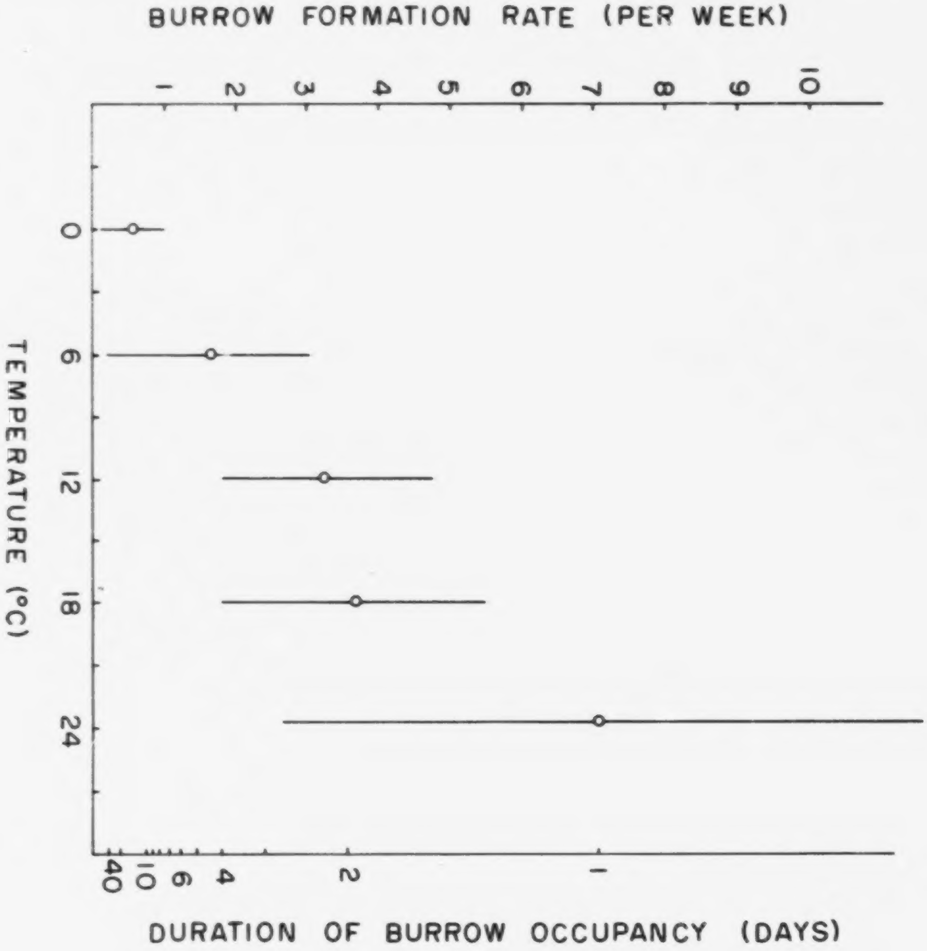

Figure 20-7. Relationship between Nephtys incisa burrow formation rate and burrow occupancy to temperature.

NOTE: Mean and range observed indicated by -0- (range limits refer only to burrowing rate axis). N = 6 for each temperature level.

Burrow-Sediment Relations

The sediment color along present and past burrows was also useful in semi-quantifying the role of *N. incisa* in 1) aeration of sediment and 2) in increasing the effective surface area of the sediment-water interface into the benthos. There is always some oxygen diffusion across any sediment-water interface, assuming the overlying water is oxygenated. Wherever burrows penetrate the sediment and are irrigated with oxygenated water, a halo of light brown or yellow oxygenated sediment soon develops around the burrow. The transition of yellow-brown to black, 2-5 cm deep, approximates the limit of oxygen penetration. Thus an increase in "aerobic" sediment is described by the expression:

$$V_{\text{halo}} = V_{\text{burrow-halo system}} - V_{\text{burrow}}$$

This volume was estimated here by measuring the light brown oxidized zone visible against the thin, sediment-filled glass aquaria using planimetry. This subsurface oxygenation persists for some time after a worm abandons the burrow, since efferent oxygenated irrigation water typically continues to course through old burrows. The rate of increase in sediment aeration following introduction of a single worm in a thin aquarium at 18°C is summarized in Figure 20-8. The 2-5 mm thick "aerobic" halo is continuous with and as thick as the aerobic zone at the sediment-water interface (see Figure 20-3). The dotted line in Figure 20-8 represents the depth of aerobic sediment in an aquarium without any worm present, that is, the aerobic zone at the sediment surface. Any increase above this level represents that resulting as a consequence of burrow irrigation activities. By comparing the aeration rates depicted in Figure 20-8, it is apparent that the slope of the curve increases with water temperature. This rise in sediment aeration eventually levels off in time as an equilibrium develops between oxygenation of new burrow sediment and chemical reduction of oxygenated sediment along old abandoned burrows. Figure 20-8 summarizes the relationship between temperature and rate of *Nephtys* sediment aeration observed in laboratory *in situ* thin aquaria. It is apparent that the extent of sediment oxygenation is positively correlated with temperature, i.e. more oxygen is delivered to deeper layers during warmer seasons. Hence, even though oxygen demand by the benthos is at its maximum during warm periods, the actual sediment aeration through *Nephtys* burrowing and irrigation can be even greater.

DISCUSSION

N. incisa burrows through sediment using adaptations previously described for other *Nephtys* and *Nereis* species (Schafer, 1962) and for *Arenicola marina* (Wells, 1952). This specialized locomotion called "bolting" refers to the head

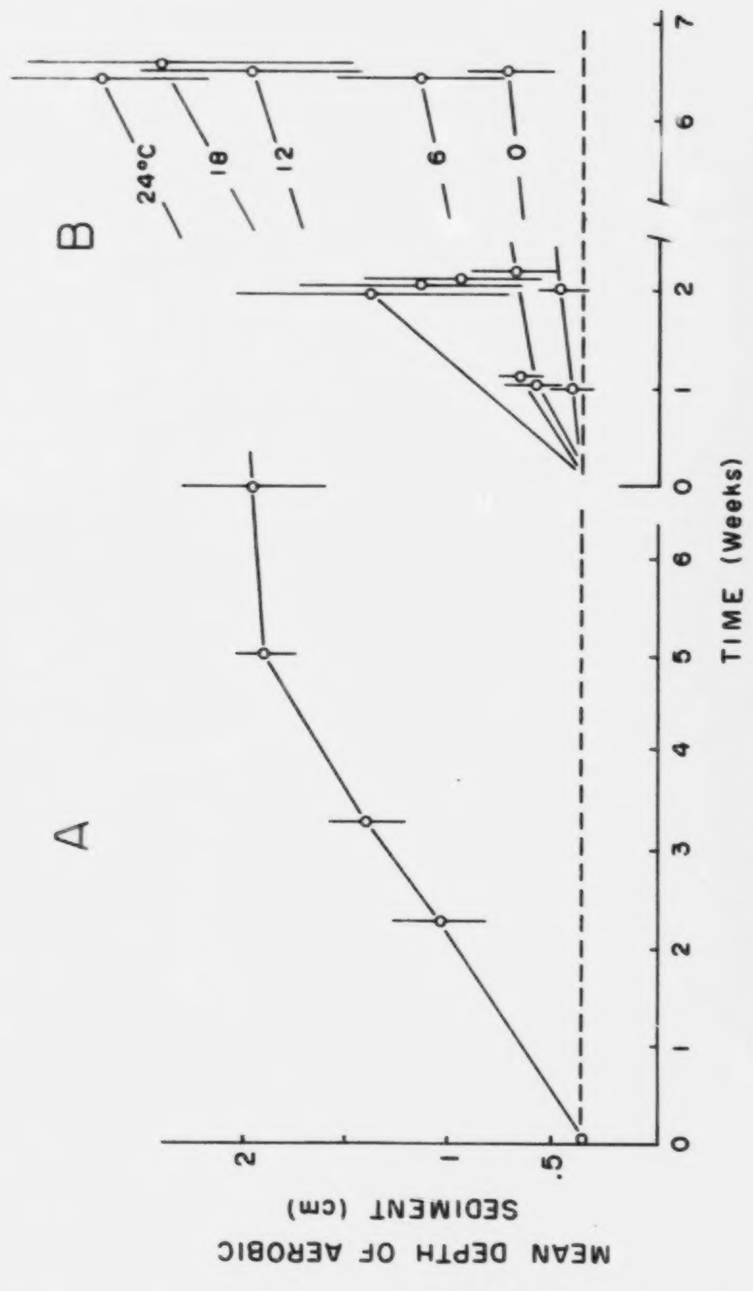

Figure 20-8. Rate of sediment aeration by *Nephtys incisa* burrow activity over time at 18°C and summary of rates of sediment aeration observed in thin-walled aquaria at 0, 6, 12, 18, and 24°C.

NOTE: The quantity of aerated sediment visible against the thin aquarium wall is expressed as mean depth of aerated sediment, although its distribution is related to burrow location as indicated in Figure 20-3.

NOTE: Dotted line represents mean depth of aerobic sediment in sediment-filled, thin-walled aquaria in absence of benthic organisms. Mean aerobic sediment depth is measured through planimetry of vertical burrowing pattern (e.g. Figure 20-3).

being forced into consolidated sediment. This is accomplished by contracting all body muscles, creating coelomic fluid pressure to expand anterior segments. The hard, bullet-shaped muscular proboscis is forced into the sediment and the pharynx finally everted when coelomic pressure reaches its peak. At this point *N. incisa* inverts its pharynx, literally sweeping an emulsion of sediment into the midgut, carried by fleshy, finger-like papillae at the tip of the everted pharynx. Immediately the posterior region of the worm crawls into the new cavity through peristaltic contractions. The bolting action is then repeated until a new burrow to the surface is complete. Other means of polychaete locomotion are also used by *N. incisa* for sediment penetration. Body undulation common to nereids is typically used by *N. incisa* to enter sediment from the water column. This more rapid sediment penetration is used only when *N. incisa* lacks a sufficient anchor on the sediment. Peristaltic locomotion in the Capitellidae is limited to movement within a burrow cavity. Both undulation and peristalsis involve a wave of segmental muscle contraction along the body from head to tail if locomotion is directed forward (Schafer, 1962).

Burrow maintenance by *N. incisa* appears limited to packing loose sediment against the burrow wall as observed in *Nereis* spp. and is termed "wallpapering" (Schafer, 1962). Burrow wall integrity may also be maintained by mucous since it is readily observed covering the setae. In addition, Schafer suggested that the iron oxides in the surrounding sediment (oxide halo) is itself a local "cementing" of sediment.

N. incisa develops temporary burrows, but unlike the continuous burrowers (e.g. *Paraonis, Heteromastis* or *Pectinaria*), *N. incisa* rapidly completes a new open burrow and then remains in it from one day during the summer to three weeks in the winter. Such a burrowing sequence suggests that *N. incisa* is abandoning discreet burrows rather than continuously meandering. The period of temporary burrow residence, occupied with feeding and irrigation activities, will be addressed in the following two papers (Davis, 1979 b,c).

The magnitude and orientation of new burrow construction may offer insight to in-sediment adaptations such as feeding or predator avoidance. In *N. incisa* the scalar values of burrowing depth and breadth appear to be strictly worm-size related, with larger worms burrowing increasingly deeper and covering greater horizontal distances. The vector measurement of sequential burrow direction appears to be random. This in contrast to other vagile polychaete burrowers. *Glycera* and *Nereis*, for instance, develop burrow galleries that maximize the worm's ability to exploit large areas of sediment surface for prey and food debris respectively. The deposit-feeders *Paraonis* and *Heteromastis* burrow in patterns termed "guided meandering". This systematic exploitation presumably minimizes repeated ingestion of sediment recently eaten. *N. incisa* shows no indication of such adaptations.

A general expression describing this depth-related burrowing intensity may be stated:

$$(1)\ I_z = \sum_{i=n}^{i=1} D_{Yi} \cdot B_T\, Y_1 + Y_2 \cdots Y_n$$

where I_z is the depth-related burrowing intensity, D_{Yi} is the mean length of burrowing by the i^{th} year class (from Figure 20-6), B_T is the temperature-dependent burrowing rate (Figure 20-7) and Y_2 is the size of the *2nd* year class.

The influence of *N. incisa* on increasing the sediment-water interface surface area is best described by computing the surface area of the burrow wall since the burrow is continuously irrigated and may be stated:

$$(2)\ S.A._L = \sum_{i=n}^{i=1} L_{Yi}\, C_{Y_1} + C_{Y_2} \cdots C_{Yn}$$

where $S.A._L$ is the burrow lumen wall surface area of a population of *N. incisa*, L_{Yi} is the mean burrow length of the Yi year class and C_{Yn} is the mean circumference of the burrow of the nth year class.

Burrow irrigation by *N. incisa* results in the oxidation of surrounding sediment. The degree of oxidation has only been expressed in a qualitative sense here. However, since the thickness of the sediment "halo" is virtually identical to the oxidized zone at the sediment-water interface, the influence of *N. incisa* on oxygenating subsurface sediment can be quantititively expressed by calculating the volume of light brown aerobic sediment surrounding the burrow for each year (size) class and extrapolating this figure over the density of that size class in a square meter of sediment:

$$(3)\ 0_{N.\ incisa} = \sum_{i=n}^{i=1} V_{halo} \cdot \left[Y_1 + Y_2 \cdots Y_n\right]$$

where 0 is the quantity of oxygen-containing sediment. V_{halo} is the volume of the oxygenated halo (Figure 20-8) and Y_1 is the density of the 1st year class in worms/m^2.

The silt-clay habitat of *N. incisa* is unique within the genus *Nephtys*, virtually singular in its sand-dwelling, predaceous life mode. This departure in sediment preference may be related to its equally unique deposit-feeding habit since silt-clay sediments are typically rich in organic matter. Motility of *N. incisa* in fine sediment can best be described as an extended period of open burrow habitation followed by its extension of the burrow developing another temporary burrow. This habit is different from infauna that constantly burrow as predators, or continual deposit-feeders, or those which reburrow only because of complete burrow destruction. Whatever the purpose(s) for high burrowing rates by *N. incisa*, reported densities of 600 – 1200 per m^2 may significantly perforate the top 10 – 15 cm of sediment during warmer months (such as suggested by model #1 above). This perforation probably results in a large increase in the surface area of the sediment-water interface (model #2). Since both present and some past burrows are irrigated, this expansion of surface area may result in a general biological model for sediment-seawater exchange, assuming a gradient as in dissolved oxygen (model #3).

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SECOND GENERATION PESTICIDES AND CRAB DEVELOPMENT

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ABSTRACT

A number of compounds have been introduced recently as potential substitutes for the traditional "hard" pesticides in the control of insect populations. Some of these compounds, juvenile hormone mimics or analogs, are intended to simulate the activity of naturally occurring juvenile hormones and prevent metamorphosis or, in the case of insect growth regulators, control differentiation or specific physiological processes at specific stages of development. Because of the phylogenetic relationship between insects and crustaceans, one might legitimately expect that those compounds which alter or interfere with the developmental pattern of insects could also have similar effects on the developmental stages of marine crustaceans.

In salinities of 20 and 35 ppt, 100 percent mortality of megalopa of *C. sapidus* occurred when exposed to 10 ppm MONO-585 while 1 ppm reduced survival from 100 percent to 40 percent. 100 percent mortality in the zoeal stages of *R. harrisii* was observed with a dilution of 1.0 ppm in reduced salinities but at 20 and 35 ppt, survival was unaffected. The concentration of 10 ppm MONO-585 was lethal in all experimental salinities. Exposure of *C. sapidus* megalopa to 0.1 ppm Methoprene resulted in reduced survival only when lower temperatures (20-25°C) were used. Juvenile crab stages I through IV were unaffected by the concentrations of Methoprene used.

The findings of these experiments and their possible significance to normal development of larvae of these two species within the natural environment are considered.

INTRODUCTION

A number of compounds have been introduced recently as potential substitutes for the traditional, "hard" pesticides (DDT, Malathion, Dieldrin, etc.) in the control of insect populations. Some of these compounds, juvenile hormone mimics or analogs, are intended to simulate the activity of naturally occurring juvenile hormones and prevent metamorphosis or, in the case of a second group, insect growth regulators, to control differentiation or specific physiological processes during development.

Several authors have reported the effects of juvenile hormone mimics on the development of insects (15, 17, 19 and others). Only a few studies, however, are reported on the effects of these compounds on other invertebrates (9, 10, 13). Of these Gomez *et al* (9) and Ramenofsky *et al* (13) first described the effect of two juvenile hormone mimics on development and metamorphosis of the cirripede, *Balanus galeatus*, and Tighe-Ford (1977) subsequently reported juvenile hormone analog effects on another species of barnacle, *Elminius modestus*. Studies on representative species of other marine Crustacea are limited, but do include the effect of two juvenile hormone mimics on larval development of the mud-crab, *Rhithropanopeus harrisii* (1, 2, 4). A study by Forward and Costlow (8) describes the manner in which one of these compounds may affect the behavior of crab larvae. Payen and Costlow (11) studied the effects of juvenile hormone mimics on gametogenesis of adult *Rhithropanopeus harrisii*.

Because of the phylogenetic relationships between insects and crustaceans, one might legitimately expect that those compounds which would alter or interfere with the developmental pattern of insects could also have similar effects during the development of marine decapods.

The present study was undertaken to further explore the effects of two compounds, methoprene (Zoecon Corporation) and MONO-585 (Monsanto Corporation) on the development of larvae of estuarine crabs. Specifically, experiments were designed to determine if these compounds would affect survival of the larvae, alter the number of larval stages, change the time required for development of all stages and metamorphosis, or affect the frequency of molting within the early juvenile crab stages after metamorphosis. A second portion of the experiment was designed to determine if effects of these compounds would be altered by changes in such environmental factors as salinity and temperature.

The two species which were selected for study were the small mud-crab, *Rhithropanopeus harrisii* (Gould), and the megalopa of the commercial blue crab, *Callinectes sapidus* Rathbun.

MATERIALS AND METHODS

Following the general rearing procedures described by Costlow and Bookhout (5) and Costlow, Bookhout and Monroe (7) ovigerous females of *C.*

sapidus and *R. harrisii* were brought in from the waters of the Newport Estuary in the vicinity of Beaufort, North Carolina, and maintained in salinities and temperatures most closely approximating those of the experimental conditions until hatching of the larvae occurred. With the experiments on *Rhithropanopeus harrisii*, the larvae were set up in separate experimental series consisting of 50 larvae per species, and maintained in temperature controlled cabinets with a photoperiod of 12 hours light and 12 hours dark, until the fourth juvenile crab stage was reached.

In experiments with *Callinectes sapidus*, which involved only the megalopa stage, larvae were maintained through the seven zoeal stages of 30 ppt, 25°C until the final zoeal molt. At that time, 20 megalopa were transferred to each of the experimental salinity and temperature conditions, and maintained in a photoperiod consisting of 12 hours light and 12 hours dark until the fourth juvenile crab stage was reached. Within each of the experimental series a control series was maintained, and at least one acetone-control series was maintained, since both methoprene and MONO-585, only slightly soluble in water, were prepared from the pure compound as an acetone stock solution of 1 ppt.

The two compounds used in this experiment were methoprene (Altosid^{R}: ZR-515; isopropyl 11-methoxy-3, 7, 11-trimethyldodeca-2, 4-dienoate) manufactured by Zoecon Corporation, Palo Alto, California, and MONO-585 (2, 6-di-t-butyl-4- (ααdimethylbenzyl) phenol) manufactured by Monsanto Chemical Company, St. Louis, Missouri.

In the experiments on *Rhithropanopeus harrisii* larvae involving MONO-585, dilutions of 10, 1 and 0.1 ppm were used in combination with 25°C, known from previous work to be the optimum temperature for development (7), and salinities of 5, 20 and 35 ppt.

In experiments with *Callinectes sapidus* megalopa, a variety of salinities, constant temperatures, and cyclic temperatures were combined with the dilutions of MONO-585 (10, 1, 0.1 ppm) or methoprene (0.1 and 0.01 ppm). These included, depending on the particular series, salinities ranging from 5 to 35 ppt and temperatures, constant or cyclic, ranging from 20°C to 35°C. The specific conditions for individual experimental series will be considered in connection with the results.

Larvae in all series were checked each day for survival and stage of development, the numbers being recorded for each experimental series. Individual megalopa were segregated in plastic compartmented boxes to avoid cannabalism, and also to facilitate recording the time of metamorphosis to the first and subsequent crab stage for each individual.

RESULTS

Effect of MONO-585 on Development

Survival of the zoea of *R. harrisii* was unaffected by the presence of 1.0 and 0.1 ppm MONO-585 when the larvae were maintained in salinities of 20 and 35 ppt (Figure 21-1). In the reduced salinity of 5 ppt, however, total mortality within the zoeal stages was observed with a dilution of 1.0 ppm, while survival at 0.1 ppm was higher than that observed for either the seawater control or the acetone control (Figure 21-1). A concentration of 10 ppm MONO-585 was lethal in all three experimental salinities and none of the zoeae developed beyond the first stage.

Megalopa of *R. harrisii* were affected by the presence of 1.0 ppm MONO-585 when combined with a high salinity of 35 ppt but survival of this last larval stage was only slightly reduced at 20 ppt (Figure 21-1). There were no reductions in survival of megalopa in 0.1 ppm, regardless of the salinity.

In those experimental salinities in which some development occurred, the time required for development from hatching to the megalopa, megalopa to the crab, and hatching to the time of final metamorphosis to the crab, was unchanged by the presence of either 1.0 or 0.1 ppm MONO-585 (Figure 21-2). The development pattern followed the sequence of four zoeae and one megalopa normally observed for *R. harrisii* and no additional or supernumerary larvae were noted.

As indicated in Figure 21-3, total mortality of megalopa of *C. sapidus* was observed in all series maintained at 5 ppt, including the control. In salinities of 20 ppt and 35 ppt, 10 ppm MONO-585 resulted in total mortality. One ppm reduced survival from 100 percent observed in the controls to 40 percent, regardless of salinity, and 0.1 ppm reduced survival to approximately 90 percent. Time for metamorphosis of the megalopa, from the final zoeal molt to the appearance of the first juvenile crab, varied from a mean of approximately 8 days to 11 days, but the presence of MONO-585 did not appear to be related to this variability (Figure 21-4).

When cultured in 5°C, 24 hour cyclic temperature (20-25°; 25-30°; and 30-35°: Costlow and Bookhout, 1971) there was no significant change in survival of control series or those series of megalopa maintained at 0.1 ppm MONO-585 (Figure 21-5). There was, however, some reduction in survival of megalopa maintained in 1.0 ppm MONO-585 coupled with all three cyclic temperatures. The greatest reduction in survival occurred when the compound was combined with a salinity of 35.0 ppt, but this effect was reduced when the cyclic temperature was increased to the maximum level of 30-35°C.

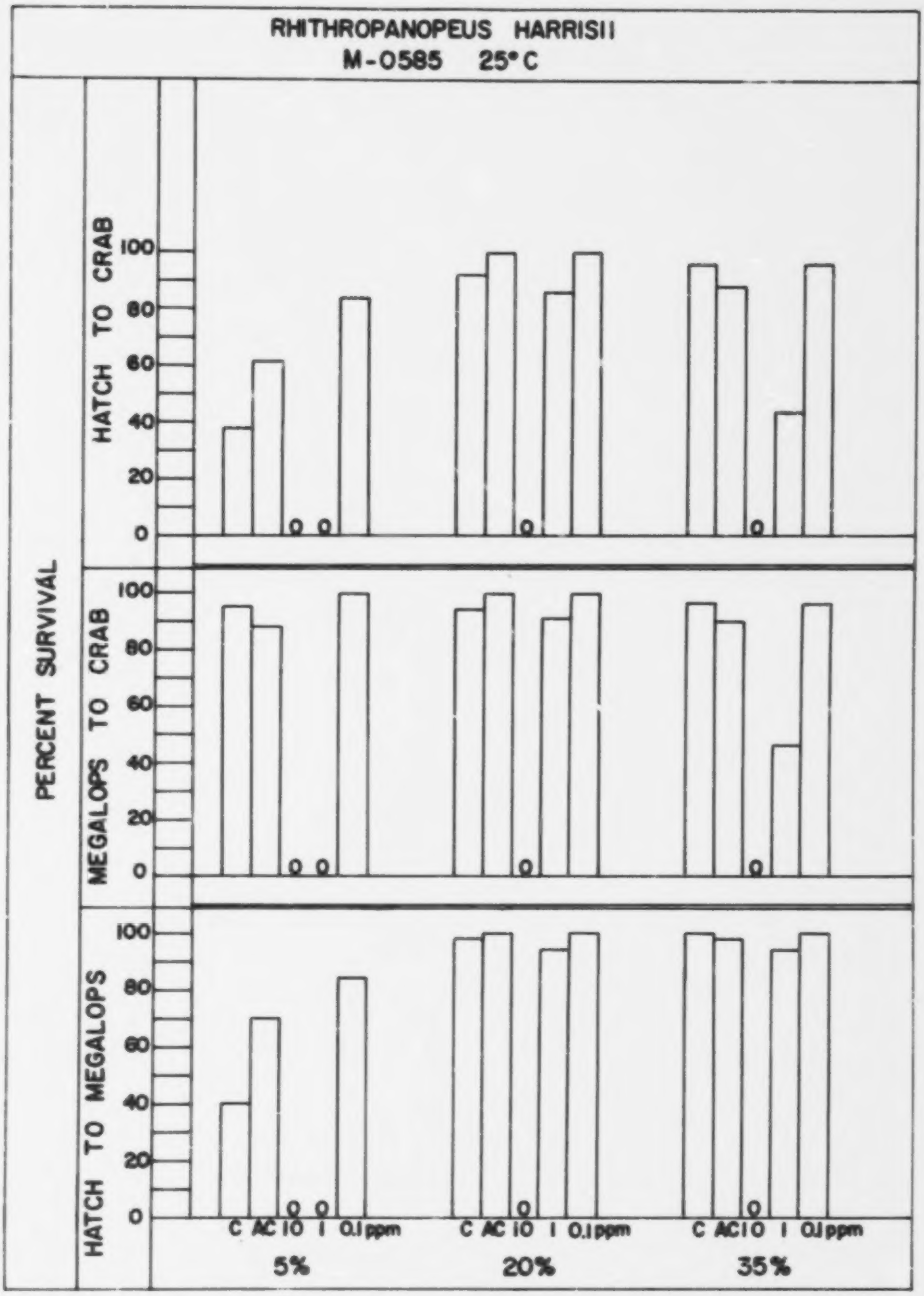

Figure 21-1. Survival of Larvae of Rhithropanopeus Harrisii Maintained at 25°C, 5 ppt, 20 ppt and 35 ppt when Exposed to Three Dilutions of MONO-585.

NOTE: C-control; AC-acetone control: 10, 1, and 0.1 ppm-dilutions of MONO-585.

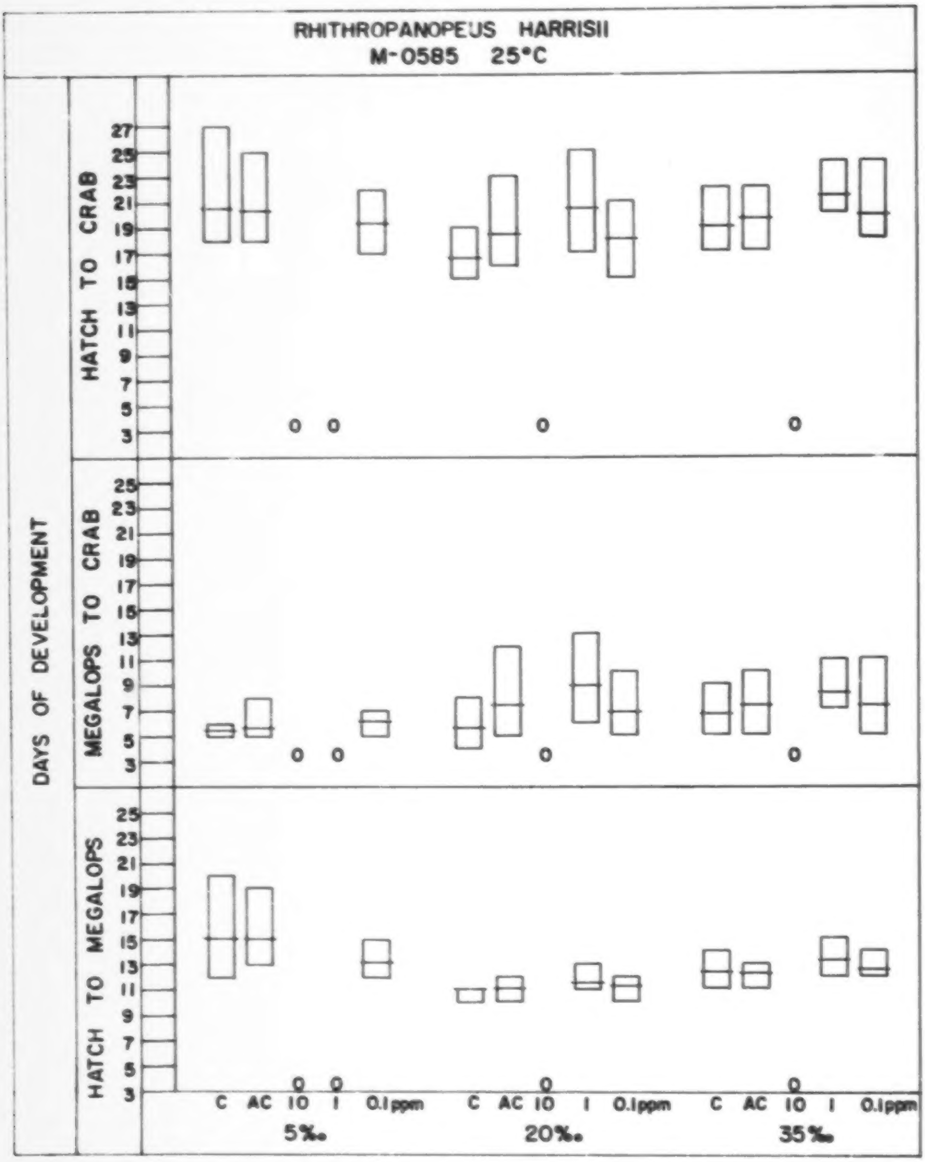

Figure 21-2. Time of Development for Zoeae and Megalopa of Rhithropanopeus Harrisii Maintained at 25°C, 5 ppt, 20 ppt and 35 ppt, when Exposed to Concentrations of MONO-585.

NOTE: C-control; AC-acetone control: 10, 1, 0.1 ppm-dilutions of MONO-585. The vertical column represents the range and the horizontal line the mean.

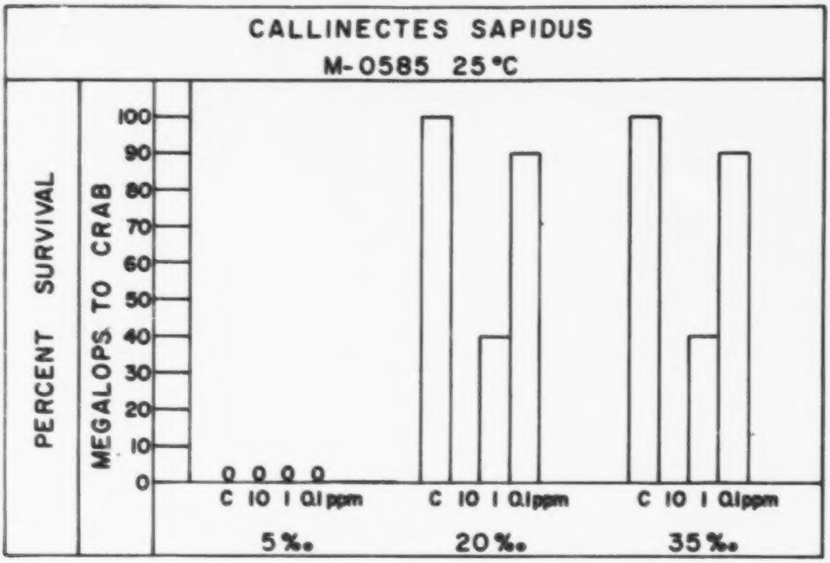

Figure 21-3. Survival of Megalopa of Callinectes Sapidus Maintained at 25°C, 5 ppt, 20 ppt, and 35 ppt, when Exposed to Dilutions of MONO-585.

NOTE: C-control: 10, 1, 0.1 ppm-dilutions of MONO-585. The vertical column represents the range and the horizontal line the mean.

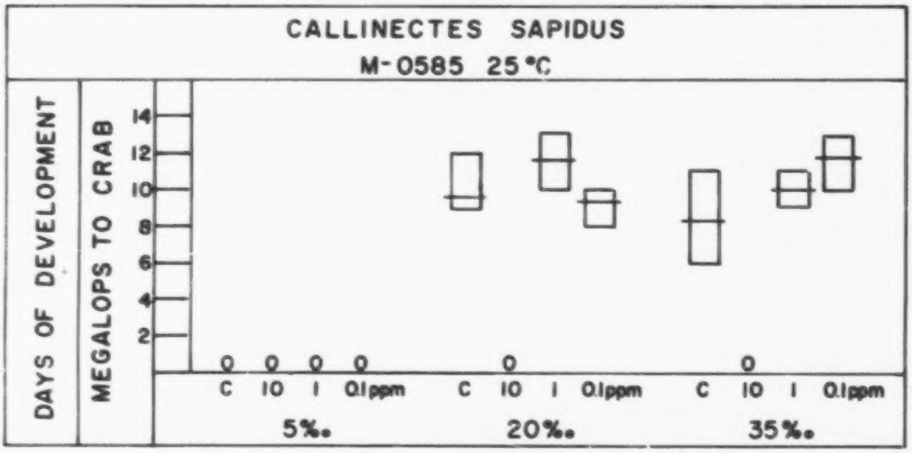

Figure 21-4. Time Required for Metamorphosis of Megalopa of Callinectes Sapidus Maintained at 25°C, 5 ppt, 20 ppt, and 35 ppt, when Exposed to Concentrations of MONO-585.

NOTE: C-control: 10, 1, 0.1 ppm-dilutions of MONO-585. The vertical column represents the range and the horizontal line the mean.

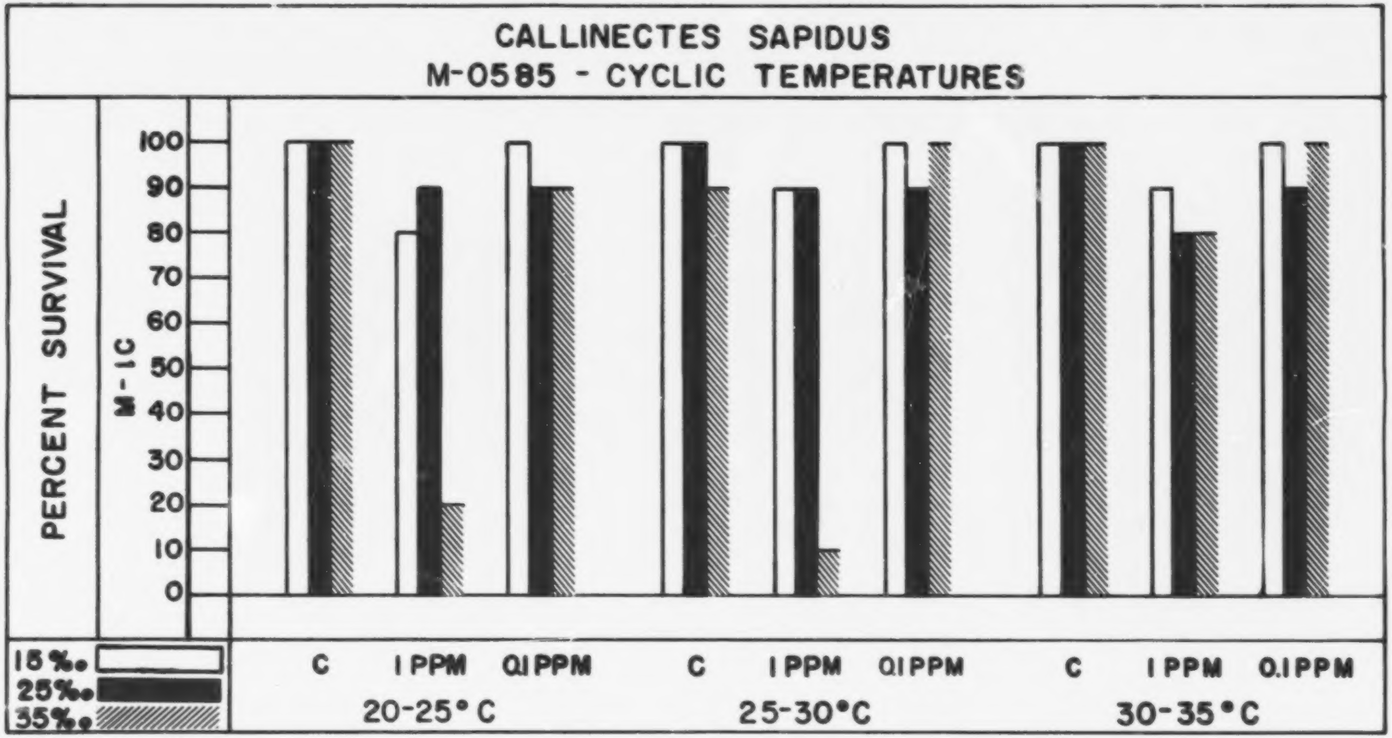

Figure 21-5. Survival of Megalopa of Callinectes Sapidus Maintained at Three Cycles of Temperature (20-25°C, 25-30°C, and 30-35°C), Three Salinities (15 ppt, 25 ppt, and 35 ppt) when Exposed to Two Dilutions of MONO-585.

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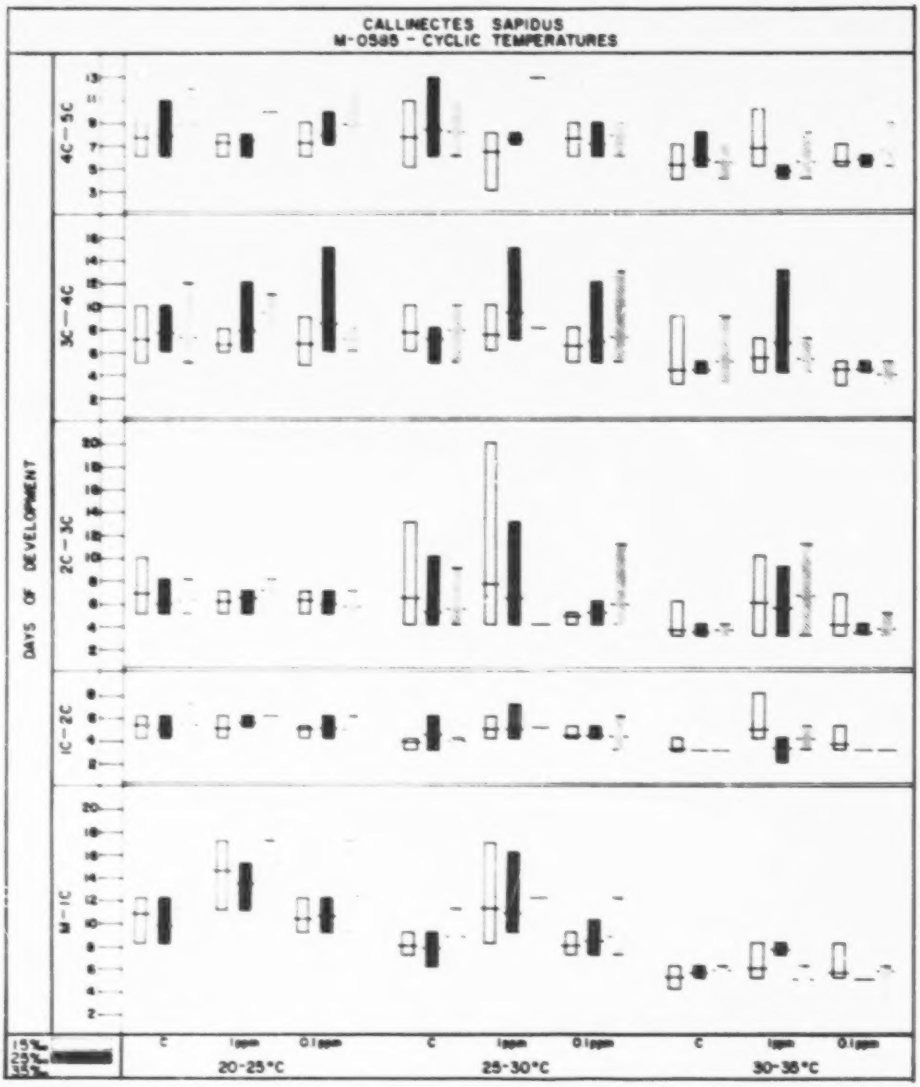

Figure 21-6. Time Required for Metamorphosis of Megalopa of Callinectes Sapidus (M-1C) and Subsequent Juvenile Molts (1C-2C, 2C-3C, 3C-4C, and 4C-5C) when Maintained at Three Cycles of Temperature (20-25°C, 25-30°C, and 30-35°C), Three Salinities (15 ppt, 25 ppt, and 35 ppt) when Exposed to Two Concentrations of MONO-585.

NOTE: The vertical column represents the range and the horizontal line the mean.

Once the megalopa have metamorphosed to juvenile crabs, there is no significant effect on survival during the subsequent four juvenile molts due to either salinity or the presence of 1.0 ppm or 0.1 ppm MONO-585 (Figure 21-6). The time required to complete the individual molts (first crab to second crab, second crab to third crab, third crab to fourth crab, and fourth crab to fifth crab) varies considerably in all experimental series (Figure 21-6). As might be expected, these same intervals were considerably reduced when the megalopa and juvenile crabs were maintained in the cyclic temperatures of 25-30°C and 30-35°C (Figure 21-6).

Effect of Methoprene on Development

Neither the megalopa nor the early juvenile crabs of *C. sapidus* demonstrated significant changes in survival when exposed to 0.1 or 0.01 ppm dilutions of methoprene combined with salinities of 15, 25 and 35 ppt, and maintained in cyclic temperatures of 25-30°C or 30-35°C (Figure 21-7). In the reduced temperature cycle of 20-25°C, however, survival was reduced from 20-25 percent in the presence of 0.1 ppm methoprene, in all salinities. Juvenile crab stages, one, two, three, and four, however, did not show any reduction in survival when maintained in these same combinations. In the same combinations of cyclic temperature, salinity, and methoprene, there is no apparent change in time required for completion of the megalopa stage or in the interval periods observed for the first and subsequent crab stages (Figure 21-8).

DISCUSSION

The relatively few studies to date on the effect of insect growth regulators on marine Crustacea have demonstrated that one may expect a variety of effects, depending upon the species and the chemical compound itself. Gomez *et al* (9) and Ramenofsky *et al* (13) found that hydroprene (Altozar^R) caused premature metamorphosis of larvae of the barnacle *B. galeatus* while a second mimic, methoprene (Altosid^R) had no effect on the time of metamorphosis, nor did it prevent settling when a proper substrate was available.

Two other analogs, farnesyl methyl ether (FME) and ethyl, 10, 11-epoxy-3, 7, 10, 11-tetramethyl-2-cis-trans-6-cis-trans-dodeca-dienoate (Ro-8-4314) were shown by Tighe-Ford (1977) to interfere with the development of *Elminius modestus* larvae, with the effect apparently related to the state of physiological development of larvae at the time of exposure. Costlow (4) described the effects of methoprene (Altosid^R) on larvae of the estuarine mudcrab, *Rhithropanopeus harrisii* (Gould) and indicated that 1.0 ppm resulted in total mortality of the larval stages, usually within the first two days of hatching. If the larvae were maintained in salinities as low as 5 ppt, survival within the

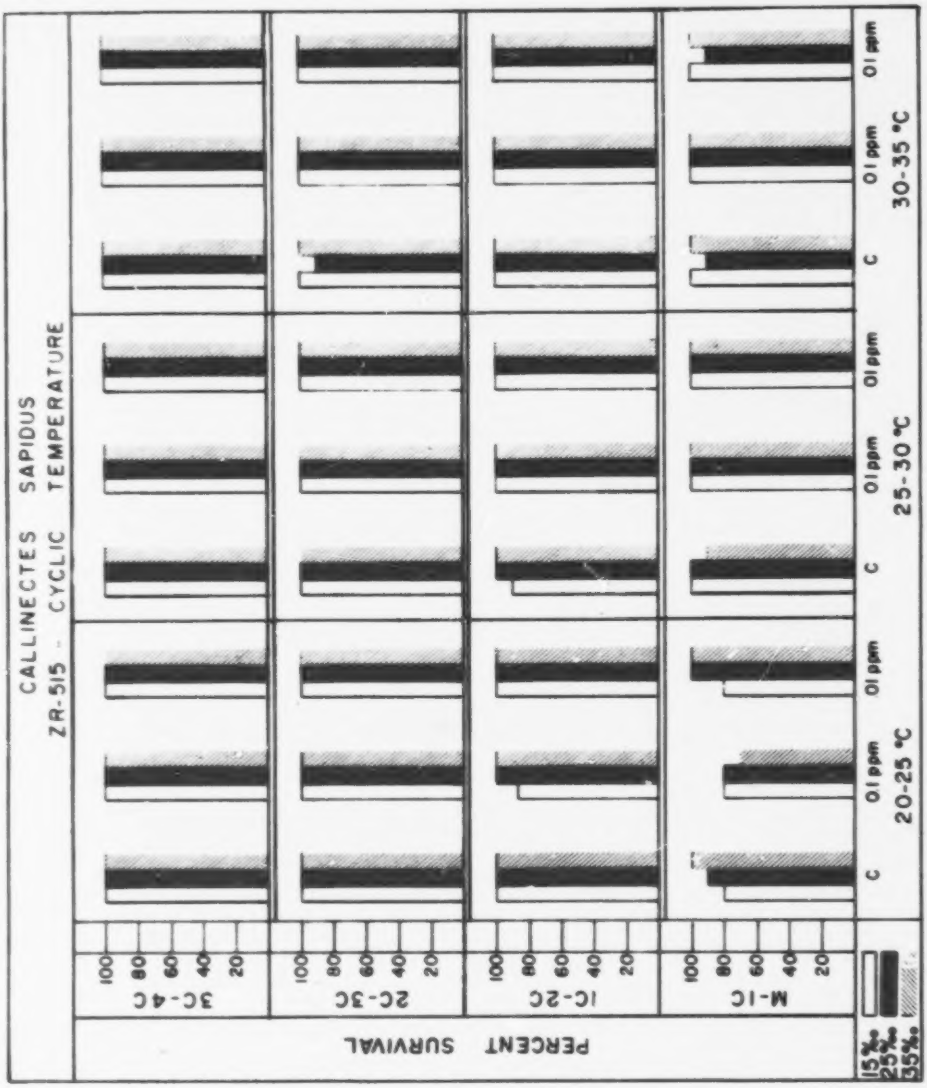

Figure 21-7. Survival of Megalopa and Early Juvenile Crabs of Callinectes Sapidus Maintained at Three Cycles of Temperature (20-25°C, 25-30°C, and 30-35°C), Three Salinities (15 ppt, 25 ppt, and 35 ppt) when Exposed to Two Dilutions of Methoprene.

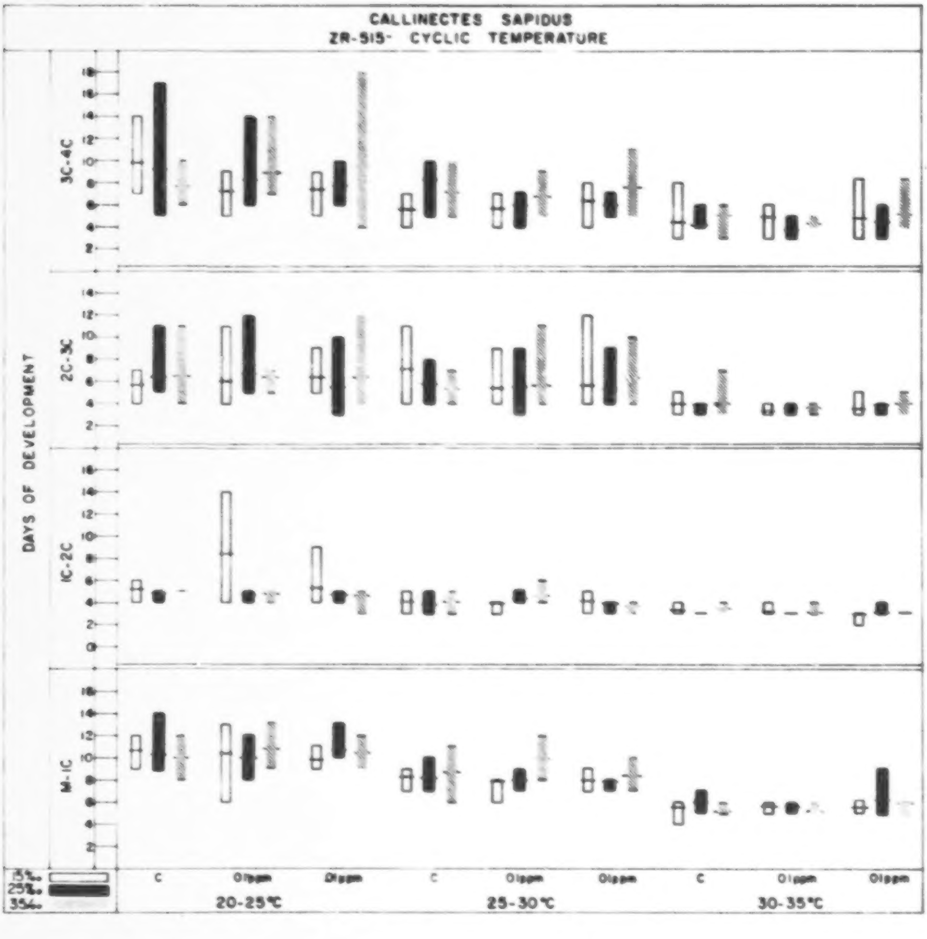

Figure 21-8. Time Required for Metamorphosis of Megalopa of Callinectes Sapidus (M-1C) and Intermolt Periods of Subsequent Juvenile Stages (1C-2C, 2C-3C, and 3C-4C) when Maintained at Three Cycles of Temperature (20-25°C, 25-30°C, and 30-35°C), Three Salinities (15 ppt, 25 ppt, and 35 ppt) when Exposed to Two Dilutions of Methoprene.

NOTE: The vertical column represents the range and the horizontal line the mean.

megalopa stage was reduced in concentrations of 0.01 ppm and 0.0001. methoprene, but there was no significant effect on the survival for larvae maintained in higher salinities. The developmental time was not significantly altered by the two lower concentrations of the compound and super-numerary larval stages were not observed in any of the experimental series. There was evidence that the early megalopa stage represented a period of extreme sensitivity to environmental stress in any form, including the presence of 0.1 ppm methoprene when combined with 5 ppt or 35 ppt (Costlow, 1977).

Christiansen, Costlow, and Monroe (1) reported a significant reduction in survival of zoeal larvae of *Rhithropanopeus harrisii* with increasing concentrations of methoprene (Altosid^R: ZR-515) and further observed an increase in the duration of zoeal stages as the concentration of methoprene was increased, irrespective of changes in temperature or salinity. Below 0.1 ppm, methoprene did not inhibit metamorphosis. The work with a second compound, hydroprene (Altozar^R: ZR-512) also resulted in a significant reduction of survival of larvae of *Rhithropanopeus harrisii*, and the first stage larvae appear to be the most sensitive stage within the four zoeae and one megalopa. Metamorphosis to the first crab stage was not inhibited at concentrations of 0.5 ppm or lower.

An additional study on the way in which a third compound, MONO-585, affected the response of larvae of *R. harrisii* to light, indicated that both swimming speed and phototaxis were altered by the presence of this compound at sublethal concentrations (8). Further information on how this general group of compounds may affect a variety of physiological and developmental processes in marine crustacean larvae, however, is needed to determine if the effects observed by previous authors are limited to the relatively few compounds and few species which have heretofore been studied.

From the present study it would appear that the compound MONO-585 is not as toxic as methoprene. Although at the concentration of 10 ppm, MONO-585 was lethal to larval stages of *R. harrisii* at salinities 5, 20 and 35 ppt, 1 ppm of this compound was only lethal when it was combined with a salinity known to represent a stress condition to the developing larval stages (5 ppt). In similar studies on the effect of methoprene on the development of *R. harrisii* (4) concentrations of 0.01 ppm and 0.0001 ppm methoprene resulted in a reduction in survival of larvae at a salinity of 5 ppt but did not significantly affect survival of larvae maintained in the higher salinities. A concentration of 0.1 ppm MONO-585 had no obvious effect on survival in any of the experimental salinities. As with experiments on methoprene, duration of the four zoeal stages and one megalopa of *R. harrisii* was not affected by the lower concentrations of MONO-585.

Previous studies on survival and length of life of megalopa of *Callinectes sapidus* (3) have indicated that they will withstand a wide range of salinity and temperature and display remarkably uniform survival in all but the lower salinities (5-10 ppt). In the present experiment, survival of the control series at 20 and 35 ppt was similar to that recorded for previous studies, but the reduction in survival of the 10 ppm and 1 ppm MONO-585 clearly indicate the toxicity of this compound to the late larval stages of the commercial crab (Figure 21-3). Total mortality was observed when the larvae were exposed to 10 ppm regardless of the salinity, and at 1 ppm, survival was reduced to approximately 40 percent, while survival at 0.1 ppm resulted in a slight reduction in survival relative to the control series (Figure 21-3). As with *R. harrisii* (4), there was no significant reduction in time required for metamorphosis, regardless of the concentration of MONO-585 (Figure 21-4).

Earlier studies on the effect of cyclic temperatures, as opposed to constant temperatures, on the survival of larvae of the mud-crab *Rhithropanopeus harrisii* (6, 16) indicated that at one particular five degree cycle of temperature, 30-35°C, a significantly higher survival could be expected relative to that observed in a constant temperature of either 30°C or 35°C. In the present study with megalopa of *Callinectes sapidus*, the only obvious effect on survival in three cycles of temperature combined with three salinities and two concentrations of MONO-585, was also associated with the high cycle of temperature, 30-35°C (Figure 21-5). Although there was no significant reduction in survival at 15 or 25 ppt combined with 1.0 ppm MONO-585, megalopa maintained in a salinity of 35 ppt, 1.0 ppm MONO-585, showed a significant reduction in survival at a cycle of 20-25°C and at 25-30°C. When the megalopa were maintained at 1.0 ppm MONO-585, 30-35°C, survival was considerably increased but, as with the study on larvae of *R. harrisii*, there is at present no obvious explanation as to how this high cycle of temperature contributes to an increase in survival of the larval stages.

Very little information is available on the way in which early juvenile stages of any crab respond to natural environmental conditions or artificial compounds which may be present within the water. From the present study it would appear that the intermolt period for the first four juvenile crabs may exhibit considerable variability, but this variability cannot be attributed to either salinity, temperature, or insofar as this experiment is concenrned, the presence of sublethal concentrations of either MONO-585 or methoprene (Figures 21-5 and 21-7).

A broad range of questions remains concerning the physiological response of many crustacean larvae and adults to the juvenile hormone mimics and insect growth regulators. Nothing is known as yet as to how these compounds may be incorporated within the animal, or the way in which they may further alter behavioral or locomotory patterns. While the short-term effects on development of two species of Decapoda are described in this paper, nothing is known of the way in which long-term exposure to sublethal concentrations through a number of successive generations may contribute to mutagenic effects. Within the realm of the chemistry of these compounds, a number of questions also remain unanswered. Several authors have described the rate at which insect growth regulators degenerate within certain natural and artificial environments (16, 18) but none of these studies have investigated the degradation rate in either an estuarine or a marine environment. Most of the research which has been conducted thus far has concentrated on the effects of the intact compound, and no data appear to be available on either the breakdown products which may occur under estuarine conditions, their fate in the natural marine environment, or the way in which they may affect developmental processes of marine invertebrate animals.

Although it is clear that the juvenile hormone mimics and insect growth regulators may offer great potential as replacements for many of the more persistent pesticides, it seems equally clear that a considerable amount of research remains to be done to assure their proper use within the estuarine and coastal environments.

ACKNOWLEDGMENTS

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SOME SUGGESTIONS FOR THE COLLECTION AND ANALYSIS OF MARINE ENVIRONMENTAL DATA

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ABSTRACT

Some problems of environmental monitoring and baseline studies are briefly introduced. Use of a simple linear model is illustrated by an example which relates to determining where to establish monitoring stations along a cross-sectional area of a hypothetical estuary. A scheme for data collection and a general analysis of variance table is provided for this particular model. The methodology for analysis of a specific data set gathered at one station at regular time intervals is outlined in detail.

INTRODUCTION

There seems to be no limit to the demands for more and more data concerning the problems of prediction and protection of the marine and estuarine environments. From the volume of data being generated in some studies, the ultimate goal in monitoring and baseline establishment appears to be to measure everything, everywhere, continuously. It should be recognized that even if this virtually infinite amount of data were gathered, there is no guarantee that it would lead to complete understanding or predictive inferences from a given system. Thus, any environmental monitoring or baseline study should be practical and feasible within reasonable time and cost constraints. However, the data gathered must be accurate, pertinent to the problems at hand, concise and purposefully collected.

It is evident from the above that proper sampling of the marine environment is an important step in monitoring and impact assessment. For example, even though analytical methods for estimating certain environmental parameters may be highly accurate and precise, if the sample being analyzed is not representative, the data resulting from the analysis is relatively worthless.

A sampling program for any environmental monitoring or baseline study must consider explicitly the following items: a) the number of samples required; b) sampling frequency; c) parameters to be measured; and d) sampling locations. These items are premised on some accepted definition of the level of perturbation or impact which is ecologically significant. It is recognized that a complete environmental assessment program encompasses a relatively comprehensive characterization (physical, chemical, biological) of a system, and includes determination of the potential impacts of pollutants or environmental changes on human health and ecological systems. Lucas (8) and Eberhart (5) have provided a review of some of the difficulties in assessing impacts and have proposed some models as bases for taking and analyzing environmental data. It is our belief that programs with more limited objectives of characterizing existing conditions or identifying previously defined impacts or changes can be developed as subsets of larger environmental assessment and monitoring programs. It is our objective to briefly describe some aspects of the design and analysis of such experiments to answer some specific questions on sampling with particular reference to ichthyoplankton.

Most biotic elements of the environment are highly variable and everchanging. They must be sampled with sufficient intensity to determine the course of such changes in time and space. Empirical evidence to date concerning ichthyoplankton, as well as juvenile and adult fishes, suggests that intensive sampling over time and space may be necessary to detect reasonable changes in these populations.

STATISTICAL METHODOLOGY

There have been two general kinds of statistical methodology applied to environmental impact analysis and monitoring. They are linear model analysis and time series analysis.

Time series analysis will not be considered in detail in this report. However, it will be briefly mentioned. In time-series analysis, the correlation of a response variable to past observations is taken into account in the formulation of a statistical model. Statistical time-series analysis has been treated in several excellent books including: Anderson (1), Box and Jenkins (2), and Nelson (9). The treatment of the methodology of time-series is comprehensive in these references, and the reader is referred to them for details. Our limited understanding of the methodology suggests that relatively large amounts of data gathered at frequent and evenly spread sampling intervals are highly desirable for this methodology to be effective in most instances.

The general linear model analysis is described at various levels of detail in several statistics books, such as Cochran and Cox (3), Davies (4), Federer (6), Kempthorne (7), Sheffe (10), and Snedecor and Cochran (11). This linear model approach includes analysis of variance and regression analysis. In this approach variations in a response variable measured over time and space are decomposed into assignable sources of variations and these variations are assumed to be additive. Tests of significance, such as F-tests or variance ratio tests to determine the change in the mean value of some variable from several sample events, are based on certain assumptions such as a normal probability density and independent and homogeneous variance (10). Data from samples taken over time frequently do not conform to these assumptions. Nonstationary elements, such as seasonal or diurnal, and tidal components are often present, and the data may be highly correlated in time.

In the linear model approach time, space and sampling locations, along with replications, become a part of a planned experimental design. As a means for considering spatial and temporal variability in the linear model, the spatial and temporal distributions of biota (i.e., ichthyoplankton) are treated as a sum of responses due to assignable sources of factor levels. In addition, transformations of the response variable are sometimes used to achieve homogeneity of variances. Finally, because one can expect certain physical and biological data to be correlated, these relationships can be effectively utilized by carrying out multivariate analyses of variance and covariance analyses.

In carrying out the linear model approach to monitoring and impact assessment, the method involves formulating hypotheses or linear contrasts for carrying out the statistical tests. Among these contrasts one tests for main effects due to a defined factor and the interaction of factors of interest.

SCHEME FOR ESTABLISHING SAMPLING STATIONS

We will illustrate the use of a simple linear model by an example which relates to deciding where to establish monitoring stations along a cross-sectional area of an estuary which is of interest. For simplicity we will assume that cost constraints limit the number of samples to about 50. Our prior knowledge of the problem suggests that we should be concerned about the depth distribution of the given organism (say winter flounder larvae).

Assume that triplicate determinations of a given larval species at five locations along the cross-section of an estuary have been made at three depth levels. Since each station location has three replicate samples at each depth, the classifications "depths" and "locations" are completely crossed, while the triplicate determinations provide three replications for each combination of depth and location. In a tabulation of the data there would be three columns corresponding to depths, five rows corresponding to the five locations, and 15 cells each containing three replicate observations.

In the general case there are $x_{ij\alpha}$ observations, $i = 1,...,p$ rows, $j = 1,...,q$ columns, $\alpha = 1,...,\eta$ replicates. The model for the analysis is assumed to be:

$$x_{ij\alpha} = \mu + \xi + \eta_j + \lambda_{ij} + \delta_{ij\alpha} \cdot$$

In the above model, μ is the mean, ξ represents row effects (location variability), and η_j represents column effects (depth variability). The interaction effects λ_{ij} represent any variations which may be peculiar to a particular combination of station and depth, and the effects $\delta_{ij\alpha}$ are normally distributed random components with average value zero for each ij.

The general analysis of variance table (Table 22-1) for this particular model is shown. From this table the partitioning of the degrees of freedom can be determined as well as the appropriate tests of significance determined. Two points can be made concerning this model. They are: 1) the variation between sample locations can be estimated, and 2) the systematic variations between sample locations can be eliminated from the study of other effects, i.e. depth.

SINGLE STATION EXPERIMENT BACKGROUND

A single sample site was chosen in the West Passage of Narragansett Bay, Rhode Island to examine within station variability at a water depth of approximately 50 feet. At the time of this report, all of the detailed analyses of the data have not been completed. However, some indication of the nature of the data and the approach to be utilized are presented herein.

Sampling was conducted on four days, hourly for 24 hour periods. During each hour paired 60 cm Bongo net hauls (505 μ mesh) were made. One tow was taken in the direction of the tide and the other against it. A data set consisting of 275 samples taken on 28 June, 1 July and 7 July 1976 are being used for detailed analysis. Oblique tows of standardized length and duration were made in all cases.

The data collected were separated into two categories: 1) Physical data; and 2) species counts. The species counts were further separated into three classes: ichthyoplankton eggs, ichthyoplankton larvae, and zooplankton.

Table 22-1. General Analysis of Variance Table for First Stage Monitoring Station Selection (Basis for Table 22-1 Layout)

Source of Estimate	Sum of Squares	df	Aver. Value of Variance Estimate
Between rows	$S_i = \eta_q \Sigma_i (\overline{x}_i \cdot - \overline{x})^2$	p-1	$\sigma^2 + \eta(1 - \frac{q}{Q})\sigma_\lambda{}^2 + \eta_q \sigma_\xi{}^2$
Between columns	$S_j = \eta q \Sigma_j (\overline{x}_{i\cdot} - \overline{x})^2$	q-1	$\sigma^2 + \eta(1 - \frac{p}{P})\sigma_\lambda{}^2 + \eta_p \sigma_\eta{}^2$
Interaction	$S_{ij} = \eta \Sigma_{ij} (\overline{x}_{ij} - \overline{x}_{i\cdot} - x_{\cdot j} + \overline{x})^2$	(p-1) (q-1)	$\sigma^2 + \eta \sigma_\lambda{}^2$
Replicates	$S_{\alpha(ij)} = \Sigma_{ij\alpha} (\overline{x}_{ij\alpha} - \overline{x}_{ij})^2$	$N\text{-}p_q$	σ^2
TOTAL	$S = \Sigma_{ij} (X_{ij\alpha} - \overline{x})^2$	N-1	

In the above table:

$\overline{x_{i\cdot}}$ ≡ row mean, $\overline{x_{\cdot}}_{j}$ ≡ column mean;

$\overline{x}_{ij}$ ≡ class mean;

P and Q refer to population sizes, large with respect to q in this case.

For each sample, the number, hour, tow direction, wind speed, surface temperature, mean water column temperature (weighted), bottom temperature, water level function, light intensity, tidal factor, inside volume, outside volume, time of first oblique tow, time between tows and time of second oblique tow were recorded.

SINGLE STATION ANALYSIS

The expected sources of variability in these data are categorized and summarized as follows:

Major Source	Effect on Density Data
Seasonal	Changes in abundance over the three sampling days
Time of Day	Changes in abundance over a 24 hour period due to tidal cycle and/or water temperature, and diurnal cycle
Net Differences	Differences between the two samples from nets A and B
Avoidance	Changes in abundance over a range of tow velocities. Individual species responses may be light dependent as well

It was found from an analysis of the physical data that mean tow velocities from north and south tows (with or against tidal currents) were significantly different. Therefore it will be necessary to compare overall mean densities from north and south tows. In order to do this it is first necessary to detrend the data by means of a polynomial function, and test for the significance of the residuals from various detrending functions. After detrending the residuals can be used to test for the effect of tow direction alone.

It should be noted that if a harmonic response to time is demonstrable the response of the system variables to time cannot be effectively measured by means of a linear model.

The 275 valid observations in the data set can be classified according to certain mean effects as follows:

$$N = (n_S) \times (n_D) \div (N_N) \times (r) - m = 275$$

where:

n_S = 3 levels of seasonal effort (3 days)

n_D = 2 levels of diurnal effort (day-night)

n_N = 2 levels of net effect (2 nets)

r = 24 replicates (2 × 12) from 2 tow directions and 12 hours within each of the 2 levels of the diurnal effect

m = 13 missing samples

Due to uniform environmental conditions over the three sampling days the SD (seasonal - diurnal) interaction is not considered of interest. The second order interaction, SND (seasonal - diurnal - nets) is considered unrealistic and dropped from consideration. The general linear model then reduces to:

$$Y_{ijkl} = \mu + S_i + D_j + N_k + SN_{ik} + DN_{jk} + E_{ijkl}$$

The missing terms SD_{ij} and SND_{ijk} are pooled with the random error term E_{ijkl} According to the above model, the partitioning of the available degrees of freedom ($N - 1 = 274$) for testing results in the univariate analyses of variance Table shown as Table 22.2. The high sensitivity of the test is guaranteed by the large number of degrees of freedom available for the error term ($W = 267$). This analysis of variance model may be applied to several response variables, including eggs, larvae and zooplankton.

In addition to linearizing the periodic responses to diurnal and tidal cycles, their variance components may be removed prior to testing for these responses known to be linearly correlated with time. The approach will involve spectral analysis and periodic regression.

Table 22-2. ANOVA Table for the Analysis of the Variance Due To Selected Main Effects and Interactions, Showing the Partitioning of Degrees of Freedom for Testing.

SOURCES OF VARIANCE AND INTERACTIONS	LEVELS	d.f.	
Seasonal (Between Days)..................S	3	2	= 3 -1
Diurnal (Between Day and Night)............D	2	1	= 2 -1
Nets (Between Net A and Net B).............N	2	1	= 2 -1
Interaction (Days) × (Nets)...................SN		2	= (3 -1) (2 -1)
Interaction (Day - Night) × (Nets).............DN		1	= (2 -1) (2 -1)
Within Replicates..		267*	
Total (N - 1)..		274	

Observations: N = 275 estimates of the density of each species of fish eggs, fish larvae and zooplankton.

*The breakdown of d.f. for the "within" or error term is:

W = (r - 1) (3) (2) (2) + sd + sdn - m = 267

where r = 24 replicates; m = 13 missing observations; and sd = 2 and sdn = 2 are the d.f. corresponding to the two interactions pooled with the error term.

Detailed accounts of the results of applying these models to the single station data are now in preparation, and will be presented elsewhere.

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KANEOHE BAY: NUTRIENT MASS BALANCE, SEWAGE DIVERSION, AND ECOSYSTEM RESPONSES

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ABSTRACT

Kaneohe Bay, Hawaii, is a coral reef/estuary ecosystem presently subjected to stresses from sewage discharge and runoff. The sewage discharge is scheduled to be diverted from the bay. This "relaxation" of sewage stress will be a major ecosystem perturbation: the termination of a chronic stress which has been imposed, with increasing intensity, on the bay over the past two decades. We are treating this sewage diversion event as a controlled experiment designed to ascertain ecosystem responses to such environmental perturbation. The experiment is being performed by means of time-series field monitoring, discrete field studies, and laboratory experiments.

The stream runoff imposes short-term, catastrophic stress from fresh water and sediment influx. The sewage accounts for about 90 percent of the land-derived nutrient delivery to the bay, thus imposing an influence which stimulates biological activity.

The sediments in the bay have been a major repository for nutrients discharged into the bay; nutrient release from the sediments has been, and will continue to be, a significant process affecting the ecosystem. When the sewage stress is relaxed, planktonic responses to that event will be more rapid than benthic responses, both because the plankton are immediately responsive to the point-source sewage discharge, and because of characteristic high biomass, efficient nutrient cycling, and limited mobility of benthic organisms.

INTRODUCTION

Kaneohe Bay is a coral reef and estuary complex on the northeast (windward) coast of Oahu, Hawaii (Figure 23-1). The bay was once renowned as one of the most beautiful coral reef ecosystems in Hawaii. The reef

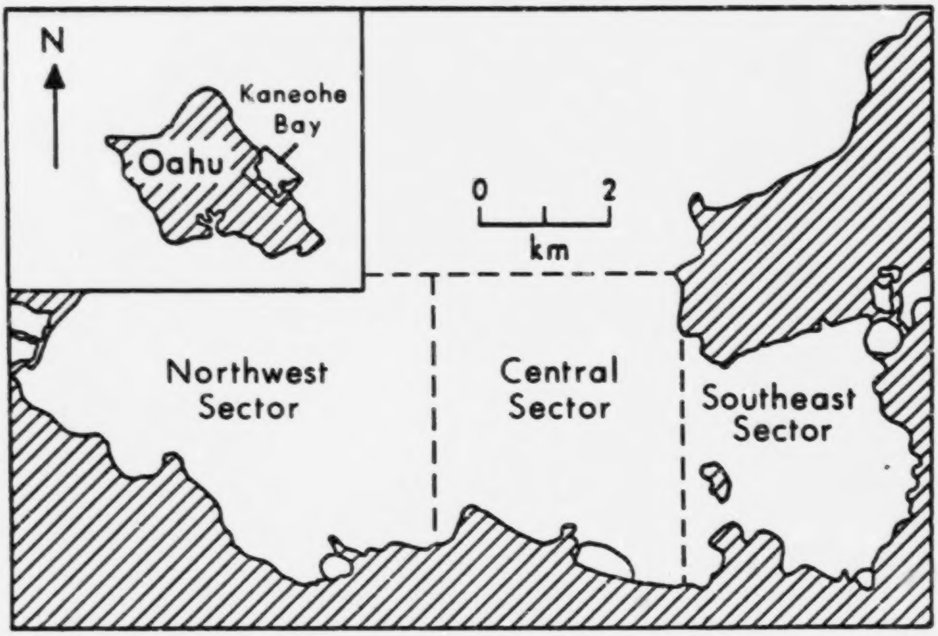

Figure 23-1. Index Map of Kaneohe Bay, Oahu, Hawaii, Showing the Locations of the Three Sectors of the Bay.

community has deteriorated, and the waters have become more turbid in response to human perturbations. Chief among these perturbations have been domestic sewage discharge and stream runoff. Both processes have been closely related to the tenfold increase of the human population in the watershed over the past three decades. Banner (2) and Smith (8) have summarized the historical conditions leading to the present environmental status of the bay.

The present stress regime is about to be drastically modified by diversion of the sewage discharge to a site removed from Kaneohe Bay. This paper discusses, from the bias of my own mass-balance approach to ecosystem analysis, interim results of a team investigation designed to ascertain ecosystem responses of Kaneohe Bay to the relaxation of sewage stress, and to derive predictive ability therefrom. The data, and many of the ideas presented here, are properly credited to other members of the research team.[1]

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[1] Working group leaders are: S. V. Smith (chemistry), E. Laws (phytoplankton), J. Hirota (zooplankton), R. E. Brock (benthos), P. L. Jokiel (microcosms). Principal cooperators from the Naval Ocean Systems Center are E. C. Evans III, J. G. Grovhoug, and R. S. Henderson.

The investigation combines field monitoring with field, microcosm, and laboratory experiments. The spatial distribution of variables in the bay is relatively well established; we have been gathering time-series data in the bay since early 1976. The outfall is due to be diverted shortly after this is written (November 1977), and we anticipate continued collection of time-series data for at least one year after the diversion.

PROJECT DESIGN

Kaneohe Bay is relatively well-described spatially, and methods by which chemical and biological characteristics of marine environments are measured are reasonably standardized. Therefore the analytical details of the study do not need discussion at this juncture. Let us instead examine the conceptual approach to this analysis.

Sewage discharge presently imposes a large and well-documented loading of biologically active materials on the ecosystem. The change of that discharge volume with time is known, and the termination date of the discharge will be a discrete, well-defined event. The discharged materials alter the water composition near the discharge sites, become incorporated into the food web, cycle within the ecosystem, and flush from the ecosystem. In addition to biostimulatory responses from the fertilization of the ecosystem, there may be responses from the loading of plant and/or animal toxins on the system. When the discharge terminates, there will be ecosystem responses as both direct and indirect ramifications of the sewage diversion.

There are three main components to the present study.

1. Routine field sampling, to document the sequence of chemical, plant, and animal changes through time. This sequence may be divided into "before diversion" and "after diversion" periods which may be compared as two distinct statistical populations of data, each of which may show seasonal or other temporal variations. The frequency of the time-series sampling is largely dependent upon the assumed or documented time scale of variability. For example, some water composition variables are measured one or more times per week, while characteristics of the benthos are measured every two months. Important adjuncts to the routine monitoring are the utilization of available Kaneohe Bay field data antecedent to our own, and use of data from environments which may be comparable to Kaneohe Bay with respect to some (but not all) of the natural and artificial ecosystem characteristics.

2. Field studies, designed to answer specific questions about the ecosystem. These studies may also establish time sequences and spatial variations in the

bay, but they are not undertaken as ongoing routine monitoring. These measurements are being made before diversion and, to the extent necessary, will be repeated after the diversion. The sampling design is modified to answer specific questions. For example: What are the relationships among variables obviously related to water clarity? While an answer can, to some extent, be extracted from routine sampling, it is more satisfactorily addressed by sampling along strong water clarity gradients which may or may not coincide with the routine sampling stations.

3. Laboratory experiments, also designed to answer specific questions about the ecosystem. Particular responses of communities within Kaneohe Bay are best addressed by controlled laboratory experiments. These experiments vary in volumetric scale from batch phytoplankton cultures in 500 ml flasks, to flow-through microcosm tanks which are 500 liters or larger in volume. The questions addressed in these simplified, but controlled laboratory experiments, cannot be easily answered under natural, and largely uncontrolled field conditions. Of course, the largest of the controlled experiments is the bay itself, a "reaction vessel" with a water volume in excess of 200 million m^3. The time scales of these experiments vary from a few days in the flasks, to months in the microcosms, and several years in the field.

In this presentation, I do not explicitly separate these various research components. Rather, I synthesize the components into our present view of total ecosystem characteristics and predicted responses to sewage diversion. This exercise is, of necessity, a preliminary analysis of our ongoing study.

MAJOR ECOSYSTEM CHANGES IN THE PAST TWO DECADES

The impact of runoff on Kaneohe Bay is largely in the form of short-term "catastrophic events." In the past 17 years, there have been three years with monthly rainfall in excess of 75 centimeters within the Kaneohe watershed (Figure 23-2). In terms of water delivery to the bay, May 1965 represented an extreme: most of the rain fell in a 2-day period and was followed by rapid runoff. A freshwater lens from that storm killed corals and other reef organisms on the fringing reef and nearshore patch reefs to a depth of up to 1.5 meters (1). The reef flats are less than 1 meter deep, so such a destructive "freshwater kill" virtually decimated the stenohaline marine organisms of the reef flats and upper portion of the reef slopes. Below about 2 meters the organisms were relatively unaffected.

Sediment loading associated with runoff has two general effects on the ecosystem, one as the material is deposited, the other as the material is in the water column. Deposition smothers reef organisms and lowers the availability

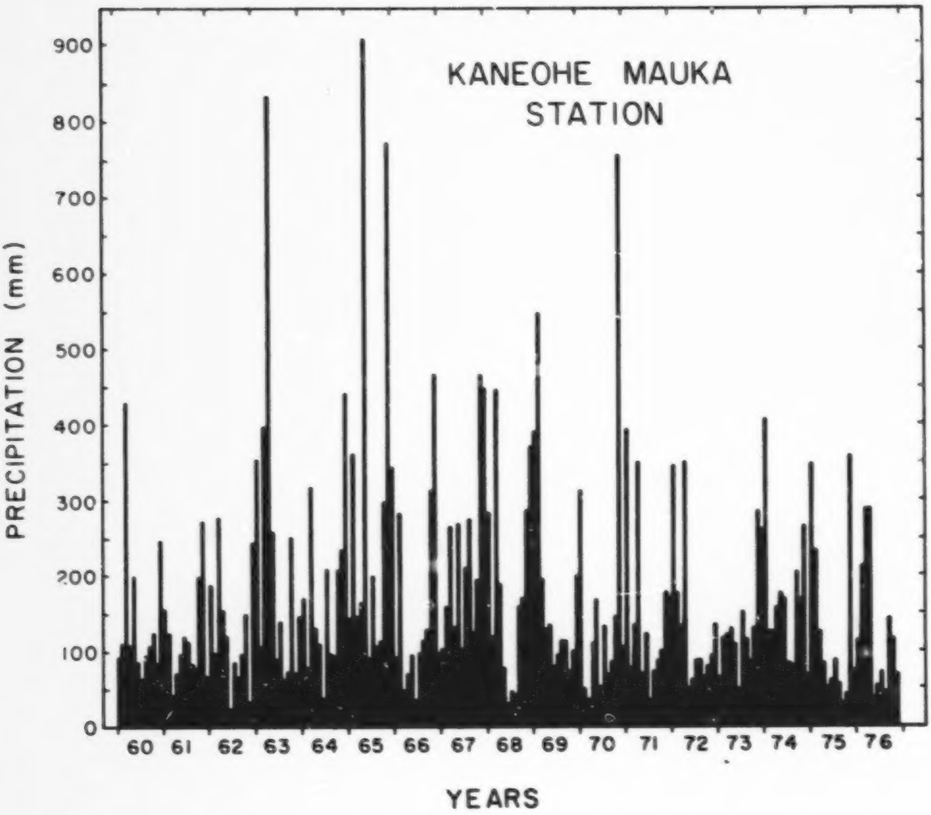

Figure 23-2. Monthly Rainfall Since 1960 at a Selected Rain Gauge in the Kaneohe Bay Watershed.

of hard substratum for settlement; particulate material in the water column lowers light and interferes with feeding mechanisms.

Nutrient loading from sewage discharge has increased about sixfold since 1963 (Figure 23-3). This increase is consistent with the previously cited rate of human population increase in the Kaneohe watershed. Virtually the entire nutrient load delivered to Kaneohe Bay is stripped from the water by biological uptake. There have been several obvious responses to the increased nutrient loading. Benthic algae are locally abundant on the reef flats and compete successfully with the corals for space on the reef slopes (2). The zone of present algal dominance on the reef slopes corresponds with the zone left undamaged by the 1965 freshwater kill. Phytoplankton standing crop and productivity are elevated above pre-loading levels; included in this high standing crop are frequent plankton "blooms" (4, plus our own data). Various

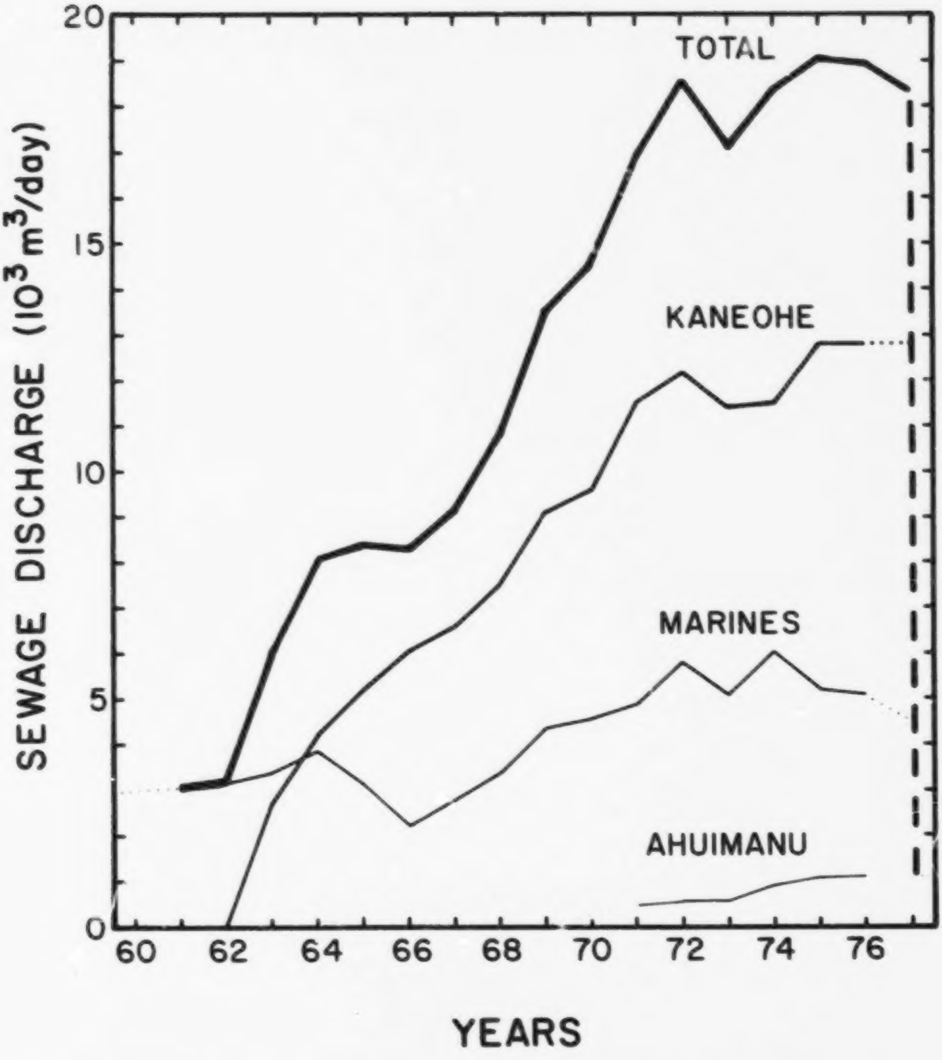

Figure 23-3. Volume of sewage discharge into Kaneohe Bay since 1960.

NOTE: The Kaneohe and Marine Corps discharge enters the southeast sector, whereas the Ahuimanu discharge flows into a stream which drains into the northwest sector.

detritivores are favored directly by organic loading associated with the sewage input, and indirectly by the products of inorganic nutrient loading (3, plus additional project data).

The relative impact of streams, rainfall, and sewage on freshwater and nutrient delivery on Kaneohe Bay can be established by a simple budgetary analysis. Sewage contributes a minimal amount of freshwater (Table 23-1); however, sewage accounts for most of the nitrogen and phosphorus delivery (Table 23-2).

The bay may thus be seen to be sporadically perturbed by fresh water and associated sediment delivery, and chronically perturbed by nutrient delivery. This latter perturbation, which has increased dramatically over the past two decades, will be terminated.

Table 23-1. Spatial Distribution of Water Inputs to Kaneohe Bay (millions of m^3/month)

	Process			
Sector	Runoff + groundwater	Rain-evap.	Sewage	Total
Southeast	2.6	-0.7	0.5	2.4
Central	1.0	-0.8	0.0	0.2
Northwest	5.0	-1.6	0.0	3.4
Total	8.6	-3.1	0.5	6.0

Table 23-2. Total Loading of "New" Nutrients on Southeast Sector (thousands of moles/day)

	Process		
Nutrient	Sewage	Streams	Total
Nitrogen	30.5	4.2	34.7
Phosphorus	3.4	0.4	3.8

NUTRIENT FLUXES

Figures 23-4 and 23-5 illustrate data for several water quality variables at three diverse locations in Kaneohe Bay. "OF" is within about 100 meters of the major sewer outfall; "S" is in the southeast sector of Kaneohe Bay; and "C" is in the central sector. Samples have also been taken from the northwest sector, where values are similar to those of the central sector.

In order for net exchange of material to occur between two water masses, a concentration gradient of that material is required. An estimate of the net mass flux between two well-mixed source waters may be obtained as the exchange volume times the concentration difference between those water masses. The concentration differences illustrated by Figure 23-4 qualitatively demonstrate

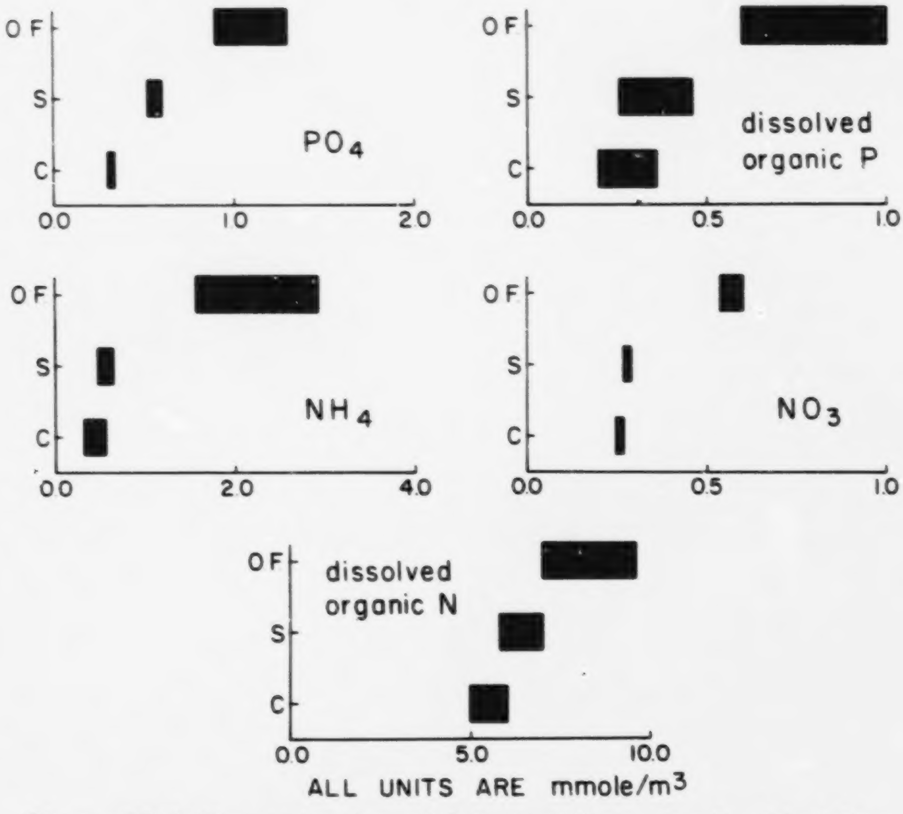

Figure 23-4. Concentration of Selected Water Quality Variables Near the Kaneohe Outfall (OF), Near the Middle of the Southeast Sector (S), and Near the Middle of the Central Sector (C).

NOTE: The bars represent the mean values ± one standard error unit.

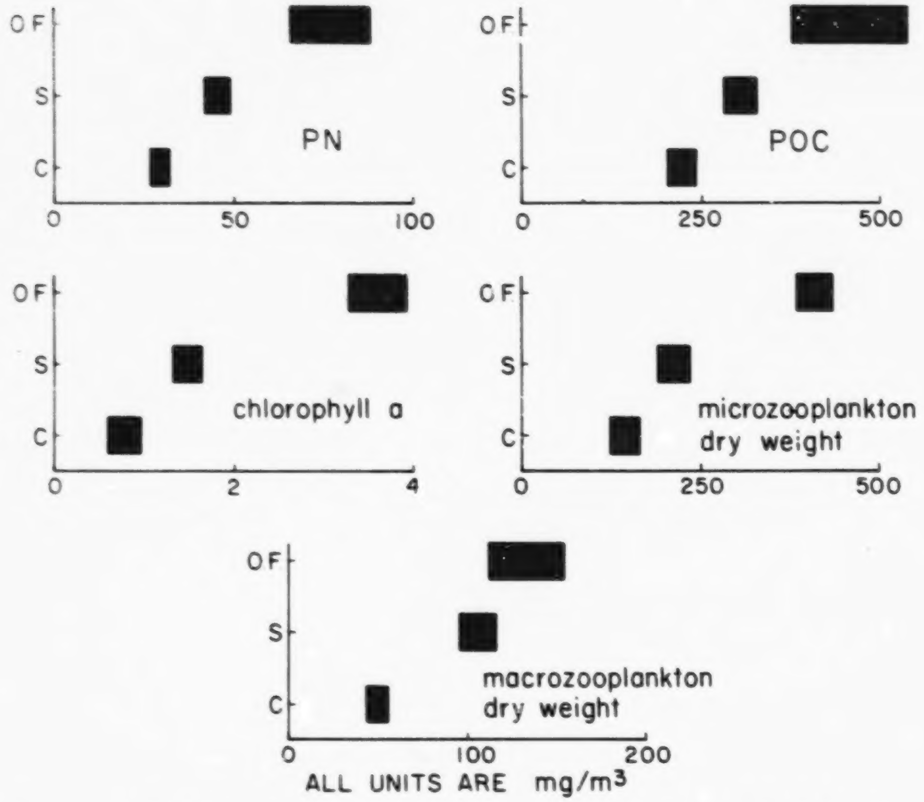

Figure 23-5. Concentration of Selected Water Quality Variables.

NOTE: Symbols explained on Figure 23-4.

the following points: (1) there is net phosphorus dispersal from the outfall to the southeast sector, and from the southeast sector to the central sector; and (2) nitrogen flux occurs from the outfall to the southeast sector, but insignificant from there to the central sector; and (3) there are also fluxes of the various particulate materials (Figure 23-5).

Quantitatively, just how significant are these fluxes? The mean residence time of water in the southeast sector of Kaneohe Bay has been variously estimated but is apparently about 20 days (10). The volume of the southeast sector is about 80 x 10^6 m^3, so the daily exchange volume is about 4 x 10^6 m^3/day. Table 23-3 summarizes nitrogen and phosphorus fluxes calculated from the concentration gradients and exchange volume, and compares these fluxes with input rates of "new" nitrogen and phosphorus. There is a dramatic imbalance between stream plus sewage input to the southeast sector, and advective output from that sector. The calculation is not entirely accurate, because some material is known to pulse from the southeast sector in a

Table 23-3. New Nutrient Input Versus Oceanic Advection from the Southeast Sector (+ is in) (thousands of moles/day)

	Process			
Nutrient	Sewage	Streams	Advection	Imbalance
Fixed inorg. N	+15.8	+2.0	-0.6	
Dissolved org. N	+13.5	+1.0	-3.6	
Particulate N	+ 1.2	+1.2	-4.5	
Total N	+30.5	+4.2	-8.7	+26.0
Inorg. P	+ 2.6	+0.2	-1.0	
Dissolved org. P	+ 0.7	+0.1	-0.3	
Particulate P	+ 0.1	+.01	-0.5	
Total P	+ 3.4	+0.4	-1.8	+ 2.0

low-density plume which flows northwestward from the sewer outfall. Nevertheless, either this simple mixing model underestimates advective losses by two-three fold, or there are additional budgetary terms to be considered.

The budget can be further amplified. There is uptake of nutrients by planktonic and benthic algae, and subsequent cycling of these particulate materials within the food web. There is fallout of particulate organic material to the lagoon floor and nutrient release from the lagoon floor back into the water column. We have obtained nutrient release rates, gathered over one year by using 1-meter diameter Plexiglas hemispheres as *in situ* incubation chambers, and we can solve for fallout by difference between nutrient inputs and outputs (Table 23-4). The advective flux of nutrients from the southeast sector equals 30-50 percent of the nutrient inputs from terrigenous sources. The fallout of particulate nitrogen substantially exceeds terrigenous nitrogen inputs to the southeast sector. The high nitrogen fallout is maintained by rapid nitrogen release from the sediments. Particulate phosphorus fallout is also high, although it does not quite exceed terrigenous inputs. As with nitrogen, the rapid phosphorus fallout is maintained in large part by nutrient release from the sediments. The steps from stream plus sewage input to particulate fallout to release from the sediments show a progressive increase in the N:P ratio (9→14→18). Material advected from the southeast sector is proportionally low in nitrogen (N:P ≈ 5), largely reflecting the virtually complete uptake of dissolved inorganic nitrogen from the water.

Table 23-4. Total Nutrient Budget for the Southeast Sector (+ is in) (thousands of moles/day)

	Process				
Nutrient	Sewage	Streams	Advection	Sediment Release	Fallout
Fixed inorg. N	+15.8	+2.0	-0.6	+14.4	0.0
Dissolved org. N	+13.5	+1.0	-3.6	0.0(3)	0.0
Particulate N	+ 1.2(1)	+1.2(2)	-4.5	0.0(4)	-40.4
Total N	+30.5	+4.2	-8.7	+14.4	-40.4
Inorg. P	+ 2.5	+0.2	-1.0	+ 0.8	0.0
Dissolved org. P	+ 0.7	+0.1	-0.3	0.0	0.0
Particulate P	+ 0.1	+0.1	-0.5(5)	0.9	- 2.8
Total P	+ 3.4	+0.4	-1.8	+ 0.8	- 2.8

(1) Particulate N & P calculated as follows. Steinhilper (9) gives sewage Part. N ≈ 1 g/m^3; flow is 18 x 10^3 m^3/day; assume N:P = 10:1.

(2) Particulate N & P in streams from stream carbon by Steinhilper (9), plus assumption of C:N:P = 100:15:1.5.

(3) Our limited data plus Hartwig's (5) data show dissolved org. N and P flux from sediment is small.

(4) Sediment resuspension of organic material excluded from calculation.

(5) Assume particulate N:P = 10:1.

The lagoon sediments are the repository for most of the "new," or terrigenous, nutrients which have been delivered to the southeast sector of Kaneohe Bay. There is a substantial cycling of nutrients between that repository and the water column, with surprisingly little loss (especially of nitrogen). This situation has been recognized on the basis of a water-column nutrient budget. We do not yet have enough sediment nutrient data to establish a quantitatively defensible sediment nutrient budget, but the nutrient level in the sediment lends qualitative support to the assertion.

PREDICTED BIOLOGICAL RESPONSES TO SEWAGE DIVERSION

The post-sewage diversion delivery of land-derived nutrients to the bay will decrease to about 10 percent of the present delivery (Table 23-2). The sediment reservoir will temporarily continue to release nutrients, but that reservoir must eventually be depleted as a fraction of the released material is constantly lost to advection. The sediment nutrient release to the water column is diffuse; while that release is sufficient to sustain a high total standing crop of plankton, that standing crop will not be as locally concentrated as the crop presently sustained by the point-source sewage input (Figure 23-5, chlorophyll).

The central and northwest sectors of the bay presently have ^{14}C productivity rates of about 5 mg c m^{-3} hr^{-1}, in comparison with about 9 mg C m^{-3} hr^{-1} in the southeast sector and 24 mg C m^{-3} hr^{-1} near the sewer outfall. There is relatively little inorganic nutrient export from the southeast sector to the other sectors (Table 23-4), so those sectors are not directly affected by the sewage. They are indirectly affected, because particulate material produced from the sewage nutrients is swept from the southeast sector and is sedimented in the other sectors, where it then releases nutrients back to the water column. Without the sewage point-source "new" nutrient input to the southeast sector, phytoplankton productivity there will stabilize near that of the other sectors. Except in the immediate vicinity of the present sewage plume, actual planktonic biomass decrease associated with the diversion should be small. Compositional shifts of both phytoplankton and zooplankton will undoubtedly occur, but we do not anticipate a significant change in the number of species present. We do anticipate a decrease in the abundance of certain meroplankton (e.g., barnacle larvae).

The benthos will also respond to sewage diversion, but more slowly than the plankton. The relatively large biomass, longevity, and relative immobility of the benthic organisms provide a substantial nutrient pool which is not as efficiently removed from the system as are suspended and dissolved materials. Some of the filter-feeding benthic animals, particularly those immediately within the sewage plume, will not survive lowered food availability. Biomass may gradually drop, but plant-animal symbioses and other relatively "tight" pathways of nutrient cycling within the benthos community are mutualistic strategies which will tend to preserve the *status quo*. Efficient internal cycling of phosphorus has been demonstrated in shallow reef benthos communities elsewhere (6,7). Nitrogen is not as efficiently retained as phosphorus (11); however, experiments we have performed suggest that, given adequate phosphorus reserves, the reef benthos community can rely on nitrogen fixation and nitrification to supplement fixed nitrogen losses (see also 12, 13).

Eventually, discrete events will disrupt portions of the benthos community. Strong onshore winds rip the benthic algae loose from the bottom, and some of that material is swept from the system. The filter feeding animals and other detritivores will largely survive until they are killed by fresh water and/or sediment inputs, although some will starve from lowered planktonic food availability.

The benthos community of the southeast sector is dramatically different from the reef community which was once found there, although historical data are insufficient to document the gain or loss of taxa. Corals, which once dominated the reefs there, as elsewhere in the bay, survive as isolated specimens. Benthic algal biomass locally exceeds pre-sewage biomass and shows large temporal fluctuation. The reef community structure has been obliterated. Benthos recovery will be back towards a coral-dominated, low-algal biomass community only if there is adequate substratum for coral settlement; if the periods between the interruptions by freshwater runoff are sufficient for community succession to proceed to the successful recruitment of corals; and if sediment nutrient release cannot maintain the high algal biomass. Banner (1) reported some coral recovery, in areas not otherwise significantly stressed, within three years of the 1965 "freshwater kill" previously mentioned. A return to coral dominance, if it ever occurs, will probably take one or more decades; shorter-term recovery patterns should indicate the direction of environmental rebound.

SUMMARY

1. The present biological structure of Kaneohe Bay may be related to the combination of catastrophic lethal events (runoff) and chronic biological stimulation (sewage discharge).

2. The nutrient deposition as particulate materials in bay sediments and subsequent release from those sediments is an important and previously undocumented part of the internal nutrient cycle within the bay. This efficient cycle allows very little nutrient loss from the bay and comprises an instantaneous nutrient delivery to the water column comparable in magnitude to the sewage input. Of course, the sediment release contrasts with the sewage input in being a diffuse, rather than a point-source, delivery of nutrients to the water column.

3. The planktonic portion of the biota can respond rapidly to alteration of environmental regimes, by virtue of advective exchange with more nearly oligotrophic waters in the absence of the point-source sewage discharge. The plankton of the bay retain relatively minor vestiges of the 1965 freshwater kill. The plankton of the southeast sector should shift rapidly to a post-sewer

composition and activity. Some qualitative aspects of the plankton community – namely the composition of the meroplankton – will have longer term residual characteristics.

4. As key components of the benthos change, their planktonic larvae should do likewise (e.g., barnacle nauplii, which dominate some plankton tows). The style of benthos succession after the 1965 freshwater kill has been influenced by sewage loading, towards a high plant and animal biomass, filter-feeding, and detritivore community. There will be a lag in the benthos response to sewage diversion. The lag will last until catastrophic events disrupt the long-term inertia maintained by the high biomass, limited mobility, and mutualism of material cycling among the benthic organisms.

5. This relatively simple examination of mass balance, hydrography, and trophic structure provides a useful basis for predicting responses of the Kaneohe Bay ecosystem to sewage diversion. As we test the predictions by post-diversion observations and continued experiments, we will be able to refine and generalize our predictive ability further.

ACKNOWLEDGEMENTS

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REPLICABILITY OF MERL MICROCOSMS: INITIAL OBSERVATIONS

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ABSTRACT

Nine microcosms at the Marine Ecosystems Research Laboratory (MERL) were run in replicate during the fall of 1976. Each microcosm tank is 5.5 m high and 1.8 m in diameter, contains 13 m^3 of water and 0.8 m^3 of sediment, and sits outdoors exposed to ambient light. Water and sediment were from Narragansett Bay. Water from the bay was run through the tanks at a rate of 330 ml per minute, resulting in a turnover time of about 27 days.

In this paper a set of the data collected during the first four months of operation is examined to discover the extent to which the microcosms replicated or diverged from each other and from the bay. Total chlorophyll *a*, nutrients, counts of individual phytoplankton species, and some other observations show that while there was considerable variability among the tanks at any given time, their overall behavior in the major features of bloom dynamics and species succession was consistent with that observed in the field.

INTRODUCTION

The development of marine microcosms has accelerated in recent years, due to an increased interest in investigating the properties of complex ecological systems, in understanding the effects of pollutants or other perturbations on such systems, and in using microcosms to carry out biogeochemical experiments (13) (3) (8) (17) (16) (10) (9) (14). The imposition of artificial boundaries and the limitation in size inevitably cause microcosms to differ from the natural systems they model. Nevertheless, the need to carry out controlled experiments on systems which represent a higher level of organization than cultures of single species has encouraged various attempts to pursue microcosm research.

At present there are no accepted criteria by which it is possible to establish whether a microcosm behaves in a way similar to the natural system it is designed to mimic, or to judge whether its behaviour is satisfactory for use as an experimental tool. A major concern should be with replicability, but one difficulty here is that nature herself is highly variable, and it is not easy to properly frame the tests to be applied.

In general, we suggest that if the enclosed ecosystems maintain similar species composition and diversity, if the metabolic rates in the systems and the major chemical fluxes and transformations are within the range of variability of the natural systems, and if the statistical behaviour of the systems is similar to that of the natural system, then it is reasonable to conclude that the major biological activities are carried on in similar ways. If so, one may have some confidence that the enclosed ecosystems are useful experimental tools.

In this paper we analyse a portion of the data obtained during the first four months of running the microcosms at the Marine Ecosystems Research Laboratory, to examine their replicability with respect to each other and to Narragansett Bay.

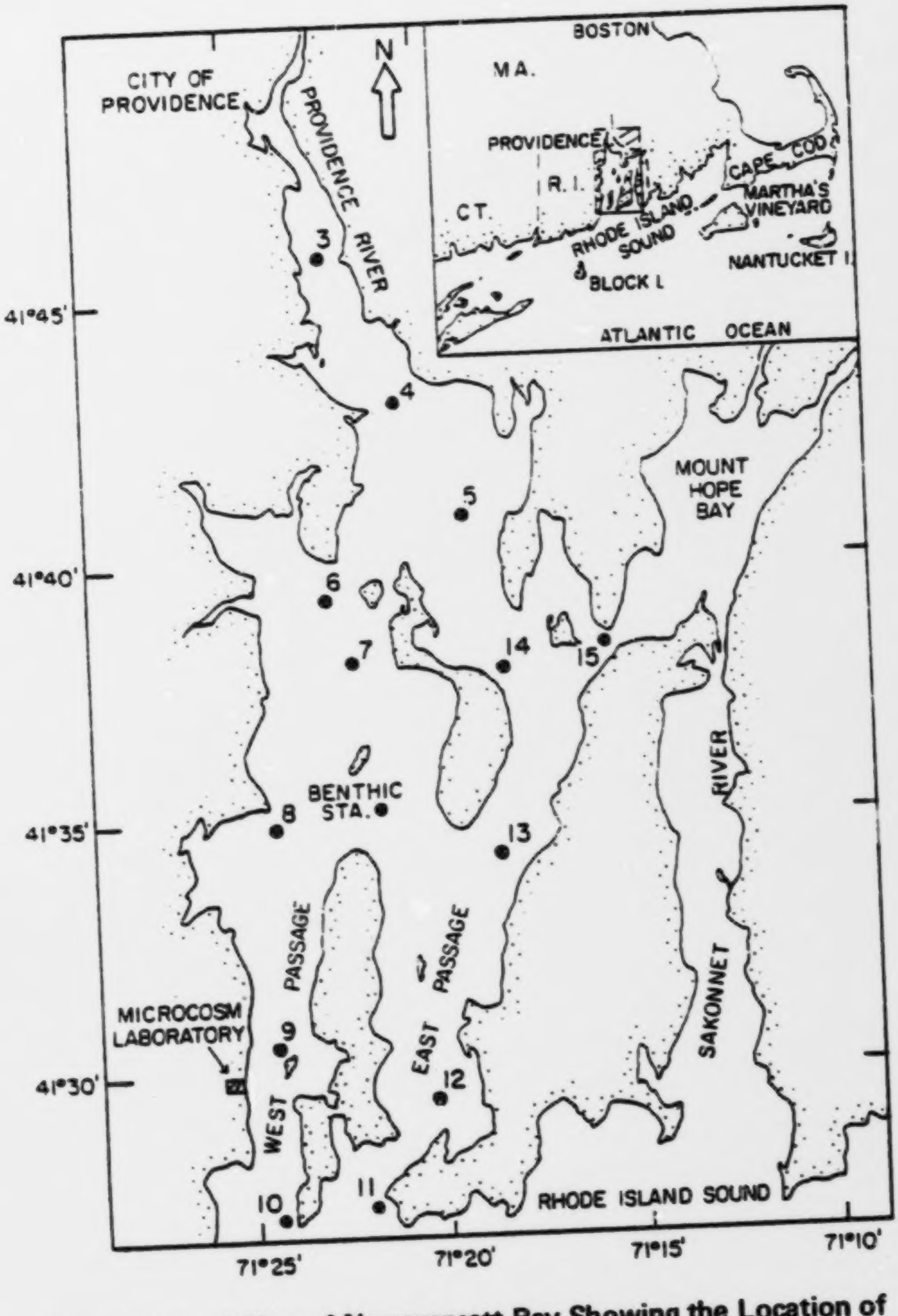
FACILITY AND PROCEDURES

Narragansett Bay

Since the microcosms to be described were designed in part to act as a model of Narragansett Bay, a brief introductory description of this bay is presented here.

Narragansett Bay is about 40 km long by 18 km wide, oriented N-S with the mouth opening into Rhode Island Sound (Figure 24-1). The presence of islands causes a complex tidal current regime and some isolation of regions. Small fresh water inputs result in a weak salinity gradient from the mouth (31 o/oo) to the northern end (∿20 o/oo). The water column is generally well mixed, although slight stratification occurs at times. The annual temperature range is from -1°C to about 25°C. Sediments are generally a mixture of silt and clay, although sand is found in some locations. Tidal currents resuspend flocculent bottom sediments in the bay, which has an average depth of about 8 m. The turnover time of the bay, based on a hydraulic model (USACE 1959) and a numerical hydrodynamic model (6) (7) is about 30 days.

Phytoplankton populations in Narragansett Bay are characterized by a winter-spring diatom bloom, followed by multiple blooms of flagellates, diatoms, and micro-flagellates in the summer. There is considerable year-to-year variation in the occurrence and timing of the various blooms.


CITY OF PROVIDENCE
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RHODE ISLAND SOUND
NANTUCKET I.
BLOCK I.
ATLANTIC OCEAN
3
4
5
6
7
14
15
MOUNT HOPE BAY
BENTHIC STA.
8
13
WEST PASSAGE
EAST PASSAGE
SAKONNET RIVER
MICROCOSM LABORATORY
9
12
10
11
RHODE ISLAND SOUND
41°45'
41°40'
41°35'
41°30'
71°25'
71°20'
71°15'
71°10'

Figure 24-1. A Map of Narragansett Bay Showing the Location of the 13 Stations Sampled During the 1972-73 Survey, the Benthic Station and the Location of MERL.

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Zooplankton of the bay are dominated by two species of *Acartia* which switch dominance depending on season. They are generally present in greatest biomass in late spring. During summer they are heavily grazed by larval fish, menhaden and ctenophores. The benthos of the bay consists mostly of heterotrophic soft bottom communities with *Mediomastis* sp. and *Nucula* sp. dominating numerically. Several areas of the bay have communities dominated by amphipods; where coarser sediments occur, large bivalves such as *Mercenaria mercenaria* and *Pitar morrhuana* may provide the most biomass.

A eutrophication gradient exists in Narragansett Bay due to sewage inputs from the Providence River (about 380,000 m^3/day). However, the lower bay is relatively clean and the water quality excellent. Average primary productivity at one station in the Bay, mostly due to phytoplankton, has been estimated to be 308 g C/yr (4) of which 45 percent may be consumed by the benthos (12).

Narragansett Bay, as well as much of the marine coastal waters of the northeast coast of the United States, is characterized by ecosystems in which most of the photosynthesis is carried out by phytoplankton, but in which the benthos plays an important part in the total cycling of energy and nutrients. The microcosm tanks were designed to maintain ecosystems functioning in a similar manner. The stirrers were designed to direct turbulent energy onto the sediments, thus effecting a resuspension of flocculent material. The tanks are exposed to natural sunlight, and their temperature regime follows that of the bay within a few degrees.

Description of microcosms

A brief description of the facility was presented by Pilson et al. (1977). Twelve fiberglass tanks are set up outdoors on land adjacent to a laboratory building. Figure 24-2 and Table 24-1 provide information on the tanks and some physical characteristics of the systems. All piping to the tanks is PVC or fiberglass, and water is pumped from a pier 30 m offshore by a diaphragm pump that appears to be non-destructive to plankton.

Sediment in the microcosms is held in fiberglass containers in the bottom of each tank. The containers were filled with sediment collected north of Conanicut Island (near "benthic station," Figure 24-1). An attempt was made to place the sediment in the right orientation in the containers, but inevitably considerable mixing occurred. Nevertheless, the major features of the benthic community in the tanks were similar to those in the bay during the period of the experiment (F. Grassle, personal communication).

Nine of the tanks were first filled during August, 1976, and maintained on a flow-through regime (330 ml/min) giving a turnover time of about 27 days.

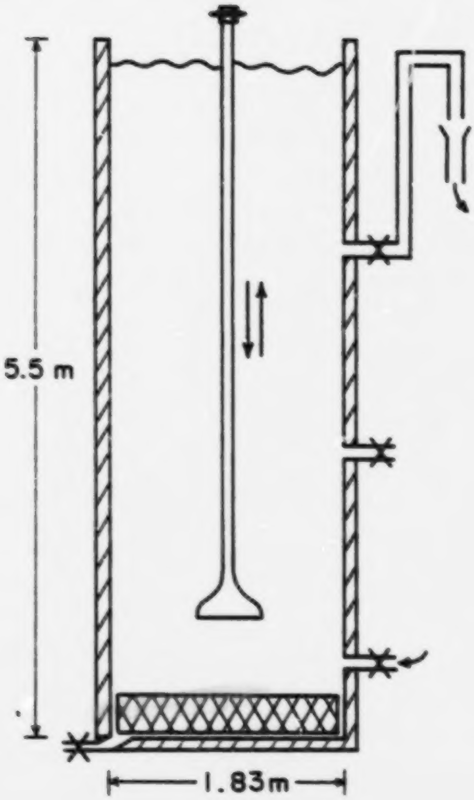

Figure 24-2. Diagram of One of the MERL Tanks.

NOTE: Each fiberglass tank is insulated and has three flanged ports on the side and one drainage port. The sediment container, also of fiberglass, contains about 30 cm of sediment. The tanks are filled through a port on the side and water exits from about 1 m below the surface through a level-control stand pipe. The depth of water is about 5 m. The mixer moves vertically through an excursion of about 60 cm with a frequency which is variable but is now set at about 5 cycles per minute.

Table 24-1. Characteristics of MERL microcosms

Tank interior diameter, 1.83 m	Mass of water, 13.3 tons
Tank interior height, 5.49 m	Area sediment, 2.52 m^2
Surface area, water, 2.63 m^2	Depth of sediment, 0.30 m
Depth of water, 4.98 m	Volume of sediment, 0.756 m^3
Volume of water, net, 13.0 m	Mass of sediment (wet), 1.10 tons
Salinity, 30 o/oo	Mass of sediment (dry), 0.568 tons

Except for some difficulties associated with the initial operations, the nine tanks were run identically during the first four months in order to assess the replicability of the systems. Some of the data obtained during this time are used in this paper to test the similarity of the tanks to each other and to Narragansett Bay.

Biological measurements used in comparisons

Table 24-2 gives a list of the data used in the analyses to be presented here. A brief description of the analytical procedures follows.

Nutrient analyses were performed using Technicon AutoAnalyser procedures somewhat modified for our purposes. Chlorophyll *a* was determined by the fluorometric method of Holm-Hansen et al. (1965).

Phytoplankton were counted on 1-ml aliquots from a pooled sample (3 depths pooled) from each MERL tank or from single samples taken at the end of the dock or in the bay. Generally the samples were counted live using a

Table 24-2. List of Measurements Yielding Data Used for Intercomparisons; Weekly Sampling During August-December 1976 Unless Noted

	MERL 9 microcosms	Narragansett Bay* a	b	c
ammonia	x	x	x	–
nitrate i nitrite	x	x	x	–
silicate	x	x	x	–
phosphate	x	x	x	–
chlorophyll *a*	x	x	x	–
phytoplankton species counts	x	x	x	x
zooplankton biomass	x	x	x	x

*Narragansett Bay

a. Data from a year-long biweekly survey made in 1972-73 at 13 stations (surface and bottom).
b. Input water to MERL microcosms, sampled from end of dock near intake line, close in time to weekly sampling of the tanks.
c. Three stations (S. Quonset, S. Patience Island, and Ohio Ledge area) sampled weekly from September to November 1976, from Durbin and Durbin (unpublished data).

Sedgwick-Rafter cell, but when this was not possible counts were done within a few days of collection on samples preserved with Lugol's iodine.

Zooplankton biomass was measured on split fractions of a pooled sample of two 1-m^3 net tows from each tank. The sub-samples were rinsed with deionized water, lyophilized and weighed.

The greatest part of the total data set consisted of phytoplankton counts. About 74 species or species categories were identified in the tanks (Table 24-3), but those that appeared five times or less were eliminated from the correspondence analysis (2). The remaining 54 species are identified in Table 24-3 by a number in brackets following the species name. Generally only 3 to 20 species were found at one time in any individual count.

RESULTS

When the microcosms were started in mid-August, 1976, the phytoplankton concentration in the Bay was decreasing after a very dense bloom (Figure 24-3). Concentrations continued to decrease until the end of August, and thereafter stayed low until the middle of November when another bloom began. The MERL microcosms followed a similar course, with the second bloom beginning somewhat earlier in some of the tanks than in the Bay. The values for chlorophyll *a* in water samples taken from the end of the GSO dock in all cases fell within the range of values plotted for the MERL microcosms.

Figure 24-4 shows a plot of the number of species of phytoplankton counted in samples from the MERL microcosms, from the end of the dock and from three other stations. In nearly every case the number of species in samples from the dock lies within the range of total species reported for the tanks. Occasionally the values for the other three stations in the Bay lie outside the range in the tanks, but the variation appears random and the data sets do not appear to be separable.

Indices of diversity and similarity were calculated using the phytoplankton species counts for the period noted. The Shannon index of diversity (Pielou 1969), which takes account of the abundance of each species, was calculated for each of the tanks and the dock for each of the weekly samples (Table 24-4). The mean value for the dock samples was higher than for the tank samples, indicating a somewhat greater phytoplankton diversity in the adjacent bay than in the tanks.

Table 24-3. List of Phytoplankton Species or Categories to which the Counts in Samples from the MERL Tanks have been Assigned

Diatoms

1. Asterionella japonica (1)
2. Attheya decora (2)
3. Biddulphia aurita
4. Ceratulina bergonii (3)
5. Chaetoceros affinis (4)
6. Chaetoceros compressus (5)
7. Chaetoceros costatus
8. Chaetoceros curvisetum (6)
9. Chaetoceros danicus (7)
10. Chaetoceros decipiens (8)
11. Chaetoceros didymus
12. Chaetoceros gracilis (9)
13. Chaetoceros lorenzianus (10)
14. Chaetoceros subtilus v. abnormis (11)
15. Chaetoceros perpusillus (12)
16. Chaetoceros sp. (solitary, small) (13)
17. Coretheron hystrix (14)
18. Coscinodiscus concinnus
19. Coscinodiscus spp. (15)
20. Coscinosira polychorda
21. Dactyliosolen mediterraneus
22. Detonula confervacea (16)
23. Ditylum brightwelli (17)
24. Eucampia zoodiacus (18)
25. Guinardia flaccida
26. Lauderia borealis (19)
27. Leptocylindrus danicus (20)
28. Leptocylindrus minimus (21)
29. Melosira nummoloides
30. Nitzschia closterium (22)
31. Nitzschia longissima (23)
32. Nitzschia seriata (24)
33. Phaeodactylum tricornutum (25)
34. Rhizosolenia delicatula (26)
35. Rhizosolenia fragilissima (27)
36. Rhizosolenia hebetata
37. Rhizosolenia setigera (28)
38. Skeletonema costatum (29)
39. Stephanopyxis turris
40. Thalassionema nitzschioides (30)
41. Thalassiosira aestivalis (31)
42. Thalassiosira decipiens
43. Thalassiosira nordenskioeldii (32)
44. Thalassiosira rotula (33)
45. Thalassiosira sp. (solitary cells) (34)
46. Thalassiosira sp. (unidentified) (35)
47. Thalassiothrix frauenfeldii (36)
48. µ pennates (37)

Flagellates

49. Amphidinium spp. (38)
50. Dinobryon spp. (39)
51. Dinophysis sp. (40)
52. Distephaneus speculum
53. Ebria sp. (41)
54. Eutreptia sp.
55. Glenodinium sp.
56. Exuviaella baltica
57. Exuviaella sp.
58. Gymnodinium simplex (42)
59. Gymnodinium sp. 1
60. Gymnodinium spp. (43)
61. Gyrodinium sp. (44)
62. Masartia rotundatum (45)
63. Olisthodiscus luteus
64. Oltmanziella sp.
65. Peridinium steinii
66. Peridinium triquetrum (46)
67. Peridinium trochoideum (47)
68. Peridinium spp. (48)
69. Prasinocladus sp. (49)
70. Prorocentrum redfieldii (50)
71. Prorocentrum scutellum (51)
72. Prorocentrum triangulatum (52)
73. Pyramimonas torta (53)
74. µ flagellates (54)

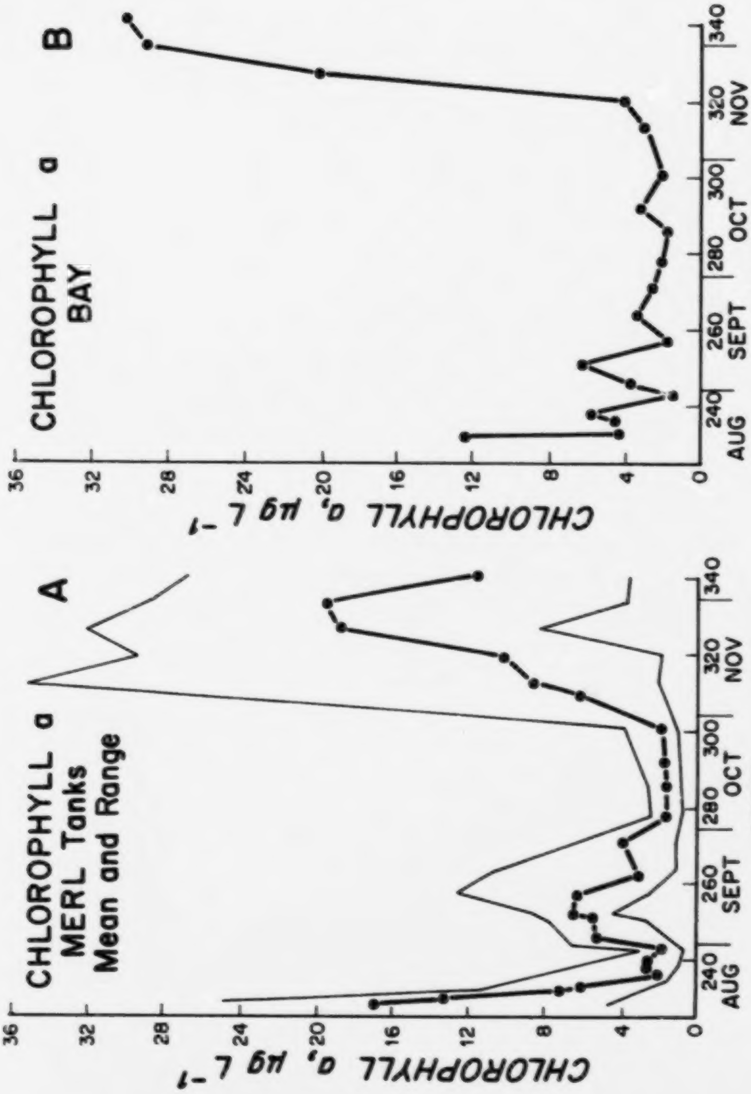

Figure 24-3. Chlorophyll a Concentration in: A. the MERL Microcosms (Mean and Range) During the Replicability Experiments; B. Narragansett Bay at the End of the Graduate School of Oceanography Dock During the Same Period of Time.

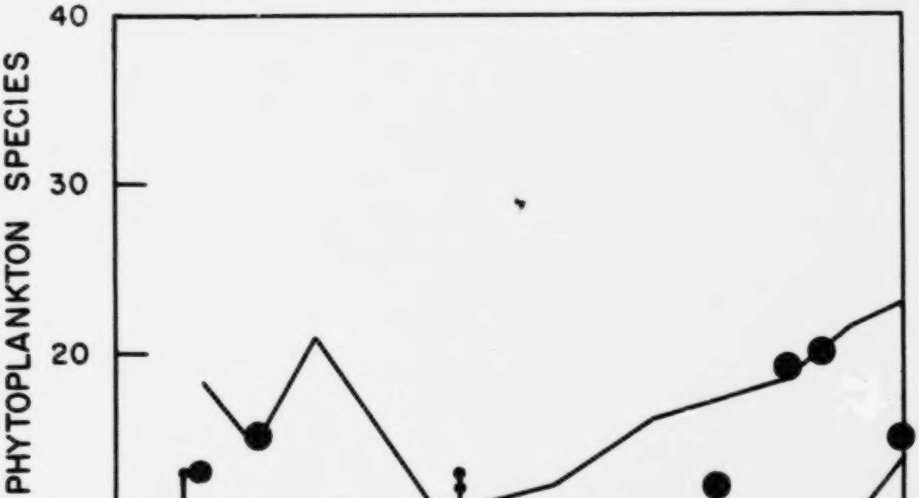

Figure 24-4. Species of Phytoplankton

NOTE: Solid lines indicate the range of the number of species of phytoplankton counted in samples from the MERL microcosms. The large opaque circles indicate the number of species from the dock samples. Three small dots connected by a vertical line indicate the number of phytoplankton species for three stations in upper Narragansett Bay (Durbin and Durbin, unpublished data).

In order to assess the similarity in types of species in the community a similarity index (13a) was calculated for each weekly data set. This index ($S = 2C/(A+B)$) is a measure of the number of species in common between two samples. Calculations were made for each tank in comparison with the dock samples, and for some tanks in comparison with each other. Some of these indices are shown in Table 24-4. In general, the inter-tank similarity indices were about the same magnitude as the bay-tank indices.

Table 24-4. Comparisons of Phytoplankton in the Microcosms and in Narragansett Bay at the GSO Dock During the Replicability Experiment.

NOTE: Similarity indices for the comparison of bay with all microcosms and for microcosm 3 with all other microcosms. Shannon index of diversity (H) for the microcosms and the bay.

	Similarity Index (S)				Shannon Index (H)	
	Bay-Tank Comparison		Tank 3-Tanks Comparison		Tank	Bay
Date	Mean	Range	Mean	Range	Mean	
Aug. 16	0.379	0.267-0.485	0.608	0.533-0.710	0.263	0.248
30	0.289	0.143- 0.944	0.617	0.500-0.800	0.021	0.270
Sept. 13	0.442	0.267-0.640	0.445	0.261-0.593	0.247	0.270
20	0.325	0.111-0.552	0.398	0.222-0.552	0.327	0.424
27	0.185	0.074-0.333	0.328	0.231-0.581	0.288	0.093
Oct. 12			0.373	0.200-0.500	0.171	–
18	0.262	0.133-0.429	0.308	0.125-0.571	0.048	0.520
27	0.286	0.190-0.545	0.458	0.316-0.667	0.209	0.391
Nov. 8	0.584	0.421-0.696	0.683	0.452-0.824	0.524	0.695
15	0.642	0.571-0.727	0.612	0.516-0.769	0.441	0.525
22	0.584	0.457-0.667	0.671	0.545-0.824	0.586	0.795
29	0.639	0.208-0.757	0.678	0.345-0.762	0.471	0.769
Dec. 6	0.660	0.467-0.788	0.669	0.588-0.757	0.558	0.810
14	0.549	0.483-0.628	0.584	0.455-0.714	0.592	0.933
22	0.530	0.414-0.648	0.584	0.455-0.709	0.599	0.807
28	0.620	0.480-0.743	0.536	0.428-0.667	0.607	0.890
Mean	0.465		0.535		0.372	0.563
Std. Dev.	±0.163		±0.130		±0.201	±0.270

While we do not have concurrent data on nutrients and chlorophyll from more than one station in Narragansett Bay, the results of a year-long survey at 13 stations in the bay taken in 1972-73 are available. Data from three of these in the lower west passage of the bay are shown in Figure 24-5. The data set from the bay is in most respects similar to that from the MERL microcosms. Chlorophyll concentrations indicated a bloom in November in both the tanks and the bay which did not occur in 1972. Ammonia concentrations in the microcosms tended to be higher than in the bay, but mean values generally fell

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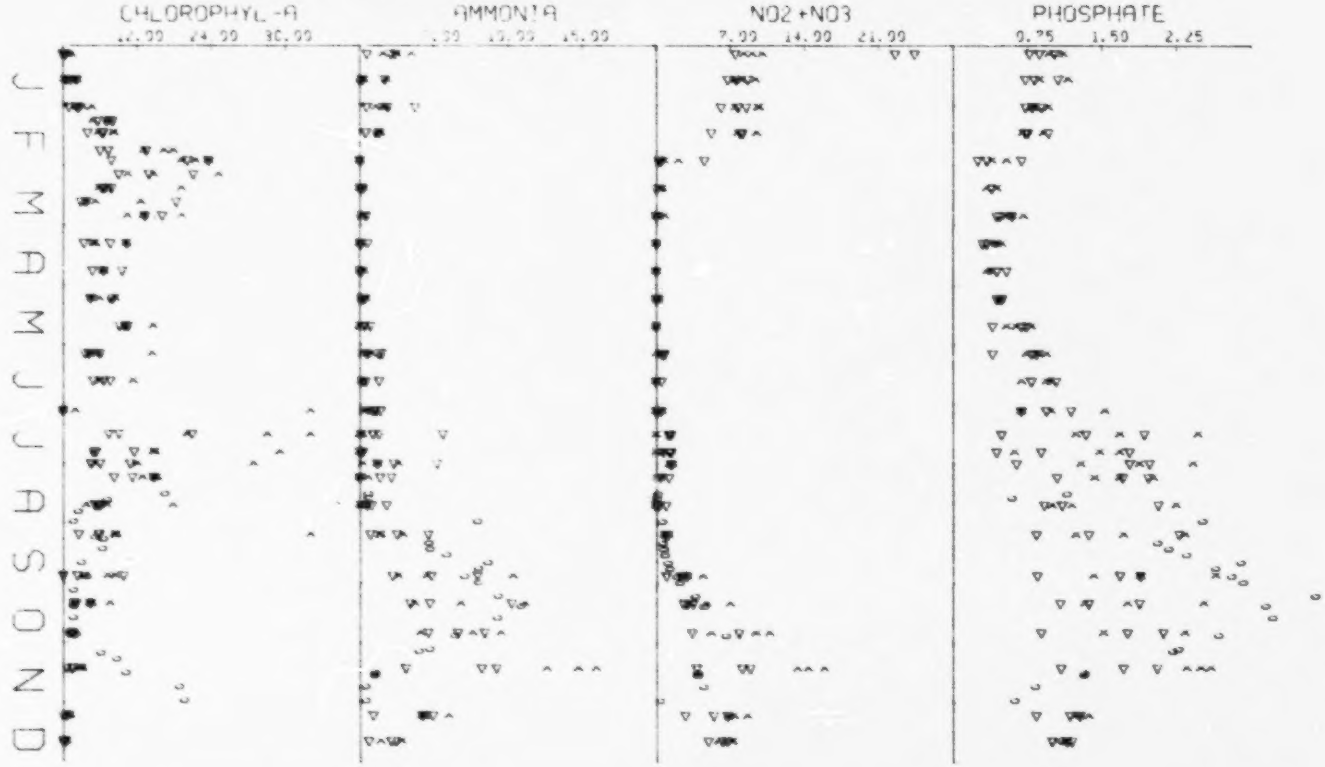

Figure 24-5. Data Collected from Surface (x) and Bottom Water (Δ) at Three Stations in the Lower West Passage of Narragansett Bay, During a Survey Carried out in 1972-73, are Compared with Mean Values from the Nine MERL Microcosms (0) During August 17 to December 31, 1976.

within the range of variability in the bay. Phosphate concentrations in the microcosms tended to be higher than in the bay during the latter part of September and October. Comparison between the chlorophyll *a* graphs in Figure 24-5 and in Figure 24-3 indicates that the timing of the phytoplankton blooms may have been different in the two years. Nevertheless, the overall behaviour of the tanks and the bay is difficult to distinguish. Figure 24-5 also gives some indication of the patchiness and other variability apparent in Narragansett Bay.

The variability and scatter of the magnitudes of individual measurements both in the bay and in the tanks show that it is difficult to distinguish tank behaviour from bay behaviour by using comparisons of single variables. In addition, various of the single variables are correlated, making statistical analysis of single variables less rigorous. Accordingly, multivariate statistical comparisons were attempted.

A stepwise discriminant analysis (11) was performed on a weekly data set from the microcosms and bay input water collected from August 17 to December 6, 1976, to observe the replicability of the microcosms. The first two axes of this analysis explained 84 percent of the variation in the data set (Figure 24-6). The first five variables in the order of their importance (nitrate-nitrite, phosphate, ammonia, silicate and zooplankton) accounted for 99 percent of the variation explained in the first two axes (Table 24-5). Chlorophyll concentration explained so little of the variation (less than 1 percent) that the analysis did not include it. Generally microcosms 1,5,6,7, and 8 were more similar to each other while microcosms 2,3,4, and 9 and bay input water showed a greater individuality. If the microcosms were exactly similar, 10 percent of each group would be classified into itself and each of the other nine groups. In fact, microcosm 3 classified 47 percent to itself, microcosm 9 classified 53 percent to itself and bay classified 44 percent to itself, indicating that these microcosms and the bay had the most individuality (Table 24-5).

The individuality of microcosms 3,9 and bay, as indicated mainly by differences in nutrient concentrations in the discriminant analysis, was not reflected in phytoplankton species as analyzed by correspondence analysis (2) (Figure 24-7). The nine microcosms were not distinguishably different in their species composition from August to December. Initially all microcosms and the bay were tightly clustered on the lower right hand side of Figure 24-7. A bloom in November was reflected in a greater variability in microcosm location and species location (left hand side of Figure 24-7), but at no time was there a characteristic species or species group which caused clearly different microcosm location.

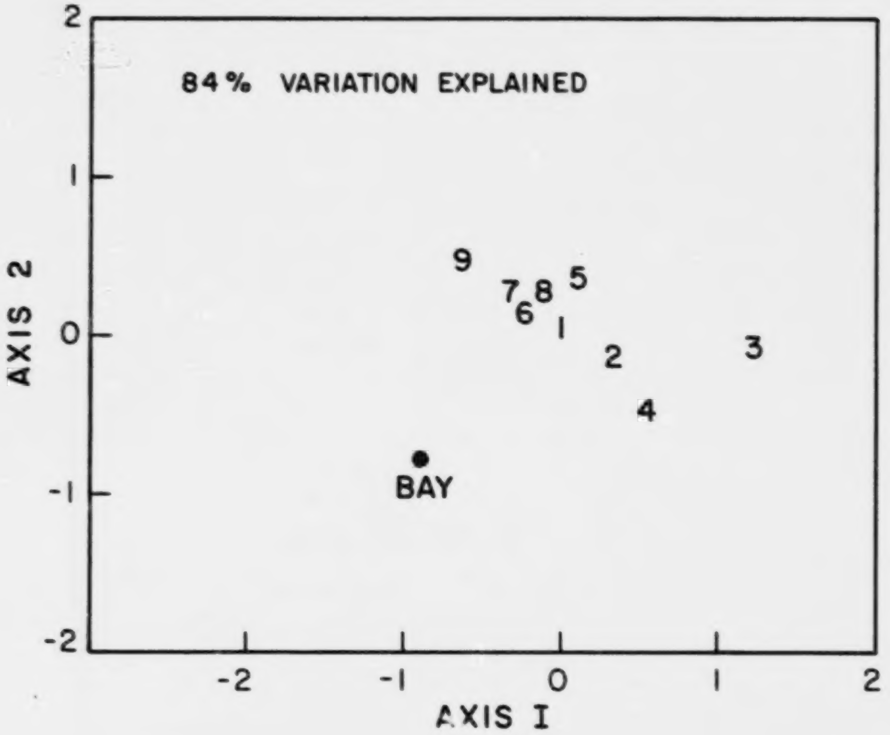

Figure 24-6. A Stepwise Discriminant Analysis of Data on Concentrations of Zooplankton Biomass, Chlorophyll, Ammonia, Nitrate Plus Nitrite, Phosphate and Silicate in 9 MERL Microcosms and Bay Input Water from August 17 to December 6, 1976, was Used to Prepare this Plot of Centroids in Reduced Space.

NOTE: Detailed information on the Analysis is presented in Table 24-5.

A comparison of microcosm data and bay data allows us to determine whether the greater variability of some of the microcosms makes them truly different from the bay. A second discriminant analysis was preformed with the same microcosm data set (nutrients, phytoplankton, zooplankton), and a similar set from the 1972-73 bay survey at 13 stations around the bay for the same period of the year (Figure 24-1, Table 24-2). In Figure 24-8, the bay stations are found positioned in a roughly linear arrangement, parallel to the first axis, which corresponds approximately to the eutrophication gradient in Narragansett Bay. Stations at the clean mouth of the bay and in the deeper east passage are positioned to the right. Stations in the west passage to the upper bay are progressively positioned to the left with the Providence River on the extreme left. All the microcosms, including those which exhibit more divergent behaviour, are positioned in mid-bay locations. Microcosm 2 appears

Table 24-5. Stepwise Discriminant Analysis of the 9 MERL Tanks and Bay Input Water from August 17 to December 6, 1976

	Percent Predicted Group Membership										
Actual Group	1	2	3	4	5	6	7	8	9	Bay	Number of Weeks
1	6.7	0	26.7	13.3	6.7	13.3	13.3	0	13.3	6.7	15
2	0	33	20.0	6.7	0	13.3	0	13.3	6.7	6.7	15
3	6.7	6.7	46.7	26.7	0	0	0	0	6.7	6.7	15
4	0	7.1	28.6	28.6	0	0	0	0	7.1	28.6	14
5	0	13.3	13.3	20.0	20.0	6.7	13.3	0	6.7	6.7	15
6	0	6.7	0	0	26.7	20.0	6.7	0	13.3	26.7	15
7	6.7	0	0	6.7	13.3	13.3	20.0	0	13.3	26.7	15
8	0	6.7	6.7	20.0	13.3	13.3	13.3	0	13.3	13.3	15
9	0	0	0	0	6.7	13.3	13.3	0	53.3	13.3	15
Bay	6.3	0	0	18.8	0	0	12.5	0	18.8	43.8	16

Percent of group cases correctly classified 27.3%. Variable not used: Chlorophyll. Total variation explained: 99%. Variables: 1 – NO_3-NO_2 (62% variation) 2 – PO_4 (23% variation) 3 – NH_4 (7% variation) 4 – SiO_4 (6% variation) 5 – Zoop. (1% variation).

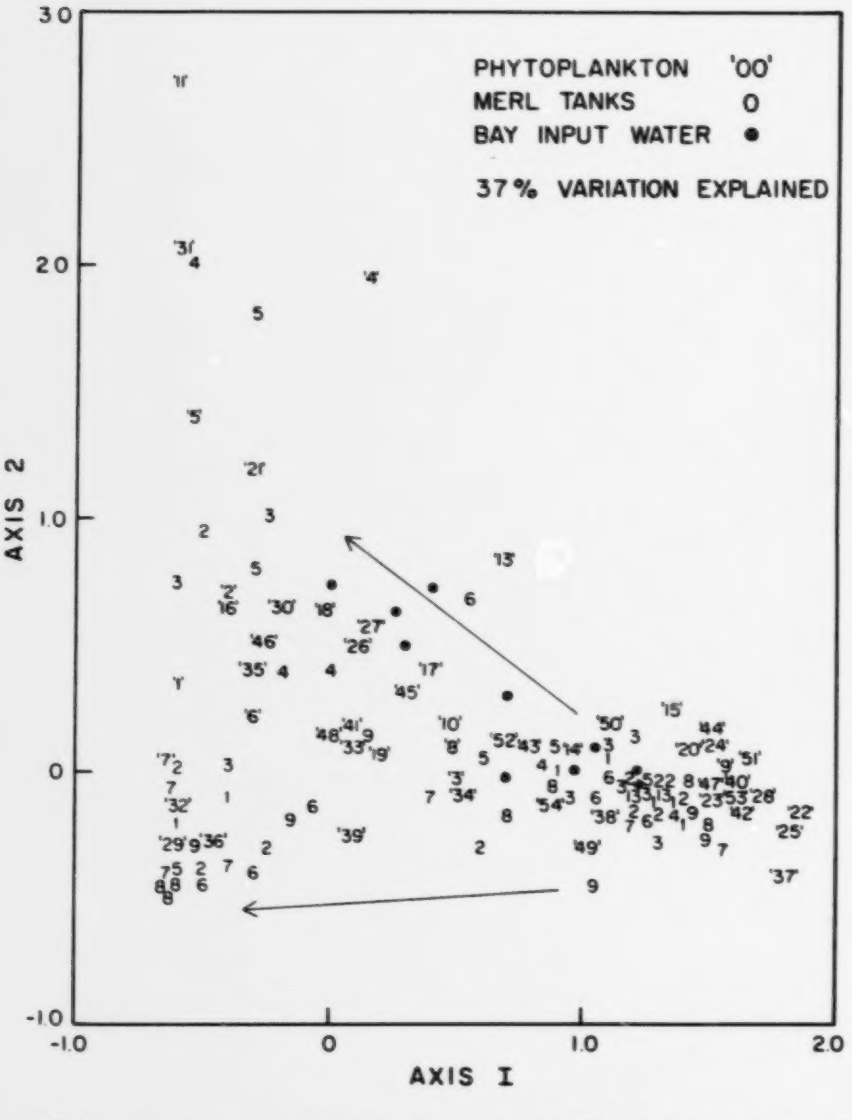

Figure 24-7. Correspondence Analysis on 49 Phytoplankton Species ('00') in 9 MERL Microcosms (0) and Bay Input Water (●) from September 14, to December 6, 1976.

NOTE: In some cases sample locations (microcosms) are closely adjacent and not all of the 10 samples per microcosm are plotted. All variable locations (phytoplankton species) are plotted as accurately as possible. Microcosm location and phytoplankton species location were closely adjacent on the lower right hand side of the figure until the first part of November when blooms occurred in the bay and in the microcosms. The arrows indicate divergence from non-bloom conditions to bloom conditions in November when variability in species composition and microcosm behavior became greater.

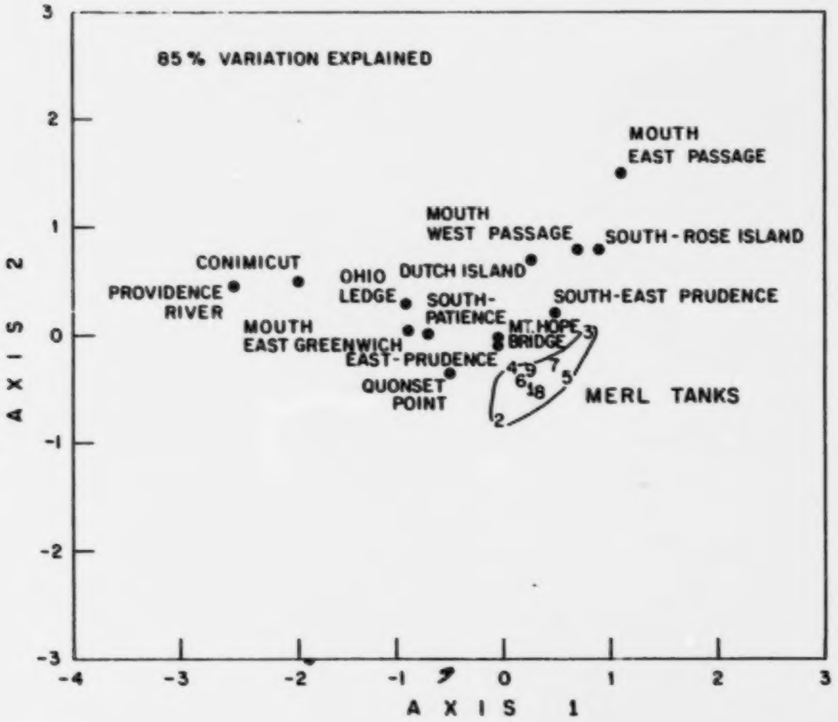

Figure 24-8. A Discriminant Analysis of Data from the 9 MERL Microcosms and the 13 Bay Stations Using the Variables: Chlorophyll, Zooplankton, Ammonia, Nitrate Plus Nitrite, Phosphate and Silicate.

NOTE: MERL data from August 17 to December 6, 1976. Bay data from 1972-1973 survey. For detailed information on the analysis see Table 24-6.

significantly different from microcosms 3, 5, 9, and microcosm 3 is significantly different from microcosm 9, but the bay stations "Dutch Island," "Southeast Prudence," "East Prudence" and "Mt. Hope Bridge," are not significantly different from any of the microcosms (Table 24-6). Other mid-bay stations are different from some of the microcosms, but no more so than they are with regard to each other.

These intercomparisons between the MERL microcosms and Narragansett Bay show that the MERL microcosms diverged surprisingly little from stations in the bay or from other microcosms, with regard to concentrations of nutrients, chlorophyll, zooplankton biomass and phytoplankton species composition during the replicability experiment.

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Table 24-6. A Discriminant Analysis of 9 MERL Microcosms and 13 Bay Stations Using Variables Chlorophyll, Zooplankton, Ammonia, Nitrate Plus Nitrite, Phosphate and Silicate from August 17 to December 6, 1976 and 1972 Respectively

PERCENT PREDICTED GROUP MEMBERSHIP

	From Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Microcosms	1	9	18	0	0	9	9	18	9	18	0	0	0	0	0	0	9	0	0	0	0	0	0
	2	0	73	9	0	0	9	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0
	3	0	10**	50	0	20	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	10
	4	0	33	0	0	11	11	0	0	22	0	0	0	0	11	0	0	0	11	0	0	0	0
	5	9	9**	36	0	27	0	0	0	0	0	0	0	0	9	0	0	0	9	0	0	0	0
	6	0	22	11	11	11	22	11	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0
	7	13	13	13	0	13	13	0	0	25	0	0	0	0	0	0	0	0	13	0	0	0	0
	8	0	0	0	0	25	25	13	25	0	0	0	0	0	13	0	0	0	0	0	0	0	0
	9	11	11**	0**	0	11	11	0	0	44	0	0	0	0	0	0	0	0	11	0	0	0	0
Narragansett Bay Stations	10	0**	0**	0**	0**	0**	0**	0**	0**	0**	71	0	29	0	0	0	0	0	0	0	0	0	0
	11	0**	0**	0**	0**	0**	0**	0**	0**	14**	43	29	0	0	0	14	0	0	0	0	0	0	0
	12	0**	14**	0**	0	0**	14**	0**	0**	14**	14**	14	14	0	0	0	14	0	0	0	0	0	0
	13	0**	0**	0**	0	0**	0	0**	0**	29**	0**	14	29	14	0	0	0	0	0	0	0	14	0
	14	0	0**	0**	0	0**	0	0	0	29	0**	0**	29	29	0	0	14	0	0	0	0	0	0
	15	14	43	0**	0	0**	0	0	0	0	0**	14**	0	0	14	0	14	0	0	0	0	0	0
	16	0	0	0	0	29	0	0	0	0	0**	0**	14**	0**	0	0	43	0	0	14	0	0	0
	17	0	0**	43	0	0	0	0	0	0	0**	0**	0**	0**	0**	0**	29	29	0	0	0	0	0
	18	0**	0**	0**	0**	0**	0**	0**	0**	0**	0**	0**	0**	0**	0**	0**	0	0	86	14	0	0	0
	19	0	0**	43	0	0	0	0	0	0**	0**	0**	0**	0**	0**	0**	0	14	14	29	0	0	0
	20	14	0	29	0	14	0	0	0	0	0**	0**	0**	0**	0	0	14	0	0	0	14	0	14
	21	14	0	0	0	0	29	0	0	0	0**	0**	0	0	29	14	0	0	0**	0**		14	0
	22	29	0	0	0	0	0	0	0	14	0**	0**	0	14	14	0	0	0	0**	0	0	14	14

** groups different at .01 level 28% correctly classified

LEGEND: 1 – 9 MERL microcosms, 10 Providence River, 11 Conimicut, 12 Ohio Ledge, 13 Month Greenwich, 14 S. Patience, 15 Quonset, 16 Dutch Is., 17 Mouth W. Passage, 18 Mouth E. Passage, 19 S. Rose Is., 20 S. E. Prudence, 21 E. Prudence, Potters Cove, 22 Mt. Hope Bridge.

DISCUSSION

These initial observations provide us with insight into the problems of running microcosms in such a way that they are analogous to some natural system. The natural system itself is highly variable and difficult to define, except within broad limits. Generally, for most of the variables measured, the values from the MERL microcosms fell within the ranges observed for adjacent Narragansett Bay. We have no evidence that the major features of phytoplankton and nutrient dynamics were different from Narragansett Bay. This lends support to the hope that the MERL microcosms will be useful experimental systems in which investigations will produce results transferable to comparable open, natural systems.

An exception to the generalizations above was the zooplankton abundance. The biomass of zooplankton in the MERL microcosms (Figure 24-9) was somewhat less than in the bay, especially towards the end of the time period considered. This factor is responsible for the microcosms lying somewhat outside the fields for the bay data shown in Figures 24-6 and 24-8, because the 1972-73 bay survey also returned somewhat higher zooplankton biomass concentrations than were found in the tanks. We believe that the tendency towards low zooplankton biomass was due to an artifact associated with the delivery of water to the tanks and that this problem will be rectified by subsequent changes to the plumbing. It is therefore premature now to dwell on the nature of the differences in zooplankton.

The general behaviour of the nutrient and the phytoplankton data sets (and, to a lesser extent, that of the zooplankton) was most reassuring. No wild excursions occurred. The variability in the microcosms was generally similar to that in mid-bay stations, and the species abundances were generally similar, taking the data as a whole. On the other hand, the quantitative variability of the tanks between themselves (as may be inferred by examination of the ranges shown in Figure 24-3) violates our usual perception of the way in which experimental systems should behave. We expect them to replicate well, so that experiments can be performed and the results have good statistical validity. The difficulty with replication of nature is that nature herself is highly variable. Working with such systems requires that large data sets be obtained, and that multivariate statistical techniques be applied to reduce these correlated data sets to manageable formats for analysis.

A possible way of assessing microcosm and natural system behaviour and developing a criterion for comparison is to calculate the generalized distance between data sets (Blackith and Reyment 1971). The assumptions of homogeneity, multivariate normality and linear correlation between variables must be met for such a technique to be rigorously applied but, as with all

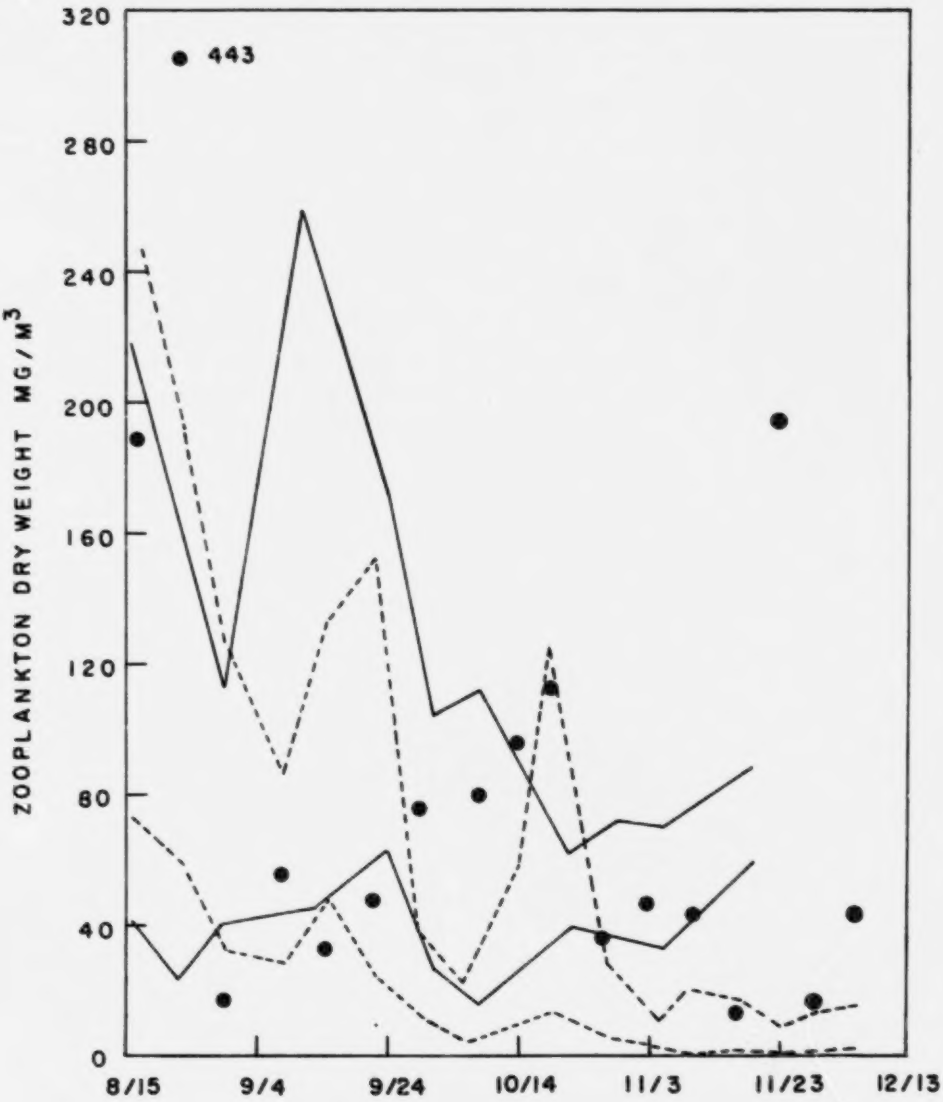

Figure 24-9. Zooplankton Biomass in the MERL Tanks and in the Bay, During the Fall of 1976.

NOTE: Solid lines give the range of data from three stations occupied by Durbin and Durbin (c.f. Figure 24-4). The solid circles give data from the GSO dock. Dashed lines give the range of data from the nine MERL tanks.

multivariate techniques, some interpretations may be explored even when the assumptions are not met. Generalized distances are determined in discriminant analysis and the maximum distances for the microcosms and the bay for the first axis in Figure 24-8 and Table 24-6 are shown in Table 24-7. These distances are much smaller among the replicated microcosms than among bay stations. It seems feasible to consider that standard generalized distances exist for natural systems, for specific variable sets, which might be compared to generalized distances which result during experiments on perturbation and subsequent recovery in microcosms.

Table 24-7. Maximum Generalized Distances and Normalized Distances Among the Microcosms and Among Bay Stations from August to December 1976 and 1972 Respectively, from the Discriminant Analysis Shown in Figure 24-7 and Table 24-6.

	D_1 max	Normalized D_1 max
Microcosms:		
2 vs 3	0.9	3
2 vs 5	0.7	2
2 vs 9	0.3	1
3 vs 9	0.6	2
Bay Stations:		
Prov. River vs mouth E. Passage	4.4	14
Ohio Ledge vs mouth E. Passage	3.0	9
Ohio Ledge vs mouth W. Passage	1.9	6

CONCLUSIONS

The nine MERL microcosms operated during the 4-month replicability study were generally as similar to each other as they were to adjacent areas of Narragansett Bay using nutrient and phytoplankton data sets for comparison.

Zooplankton abundance in the MERL microcosms was somewhat low but this was probably caused by an artifact that can be removed.

Multivariate statistical techniques seem essential to the comparison of the large and heterogeneous data sets generated in such studies as this.

The similarity in the behaviour of the microcosms and Narragansett Bay gives some confidence that these systems will be good experimental tools for ecological and biogeochemical experiements.

ACKNOWLEDGMENTS

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TURBULENT MIXING IN MARINE MICROCOSMS—SOME RELATIVE MEASURES AND ECOLOGICAL CONSEQUENCES

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ABSTRACT

The effect of turbulent water motion on pelagic organisms has seldom been studied. Nevertheless, a consideration of the theory of turbulent energy flux as well as the few bits of empirical data which do exist suggest that it may be a factor of some importance for marine plankton, and that turbulence may influence the growth, metabolism, and behavior of pelagic species as well as their spatial distribution. This paper reports the results of a series of turbulence experiments carried out over an annual cycle using small (150 l) laboratory microcosms designed as analogues of Narragansett Bay, R.I. (U.S.A.). Turbulence levels in the mocrocosms and in the natural system were characterized using conventional (neighbor diffusivity, vertical eddy diffusivity, energy flux) parameters as well as a number of relative measures of water mixing (dye dissipation, $CaSO_4$ dissolution rate, gas exchange coefficients). The response of phytoplankton and zooplankton populations to varying turbulence levels was dramatic during warmer months, but absent or unclear in winter. The results suggest that while phytoplankton may be stimulated by higher turbulence levels, at least in warmer water, the response of the zooplankton is quite the opposite during these periods. It is not clear if the response of the phytoplankton reflects a decline in grazing pressure or a real enhancement of growth. The problem is complex and deserves considerable further study both in the field and in the laboratory.

INTRODUCTION

> "... diffusion is confusion. Only Maxwell's Demon
> really knows what's going on."
>
> Akira Okubo (1971),
> Horizontal and Vertical Mixing in the Sea

A Problem of Size and Scale

The increasing use of relatively small experimental ecosystems or microcosms in marine research has raised a number of interesting questions related to the importance of size or scale in natural, as well as experimental systems. In pelagic marine environments, one of these questions for which we have very little relevant information is the importance of turbulence in the water. The relationship between turbulence and scale was first formalized by Richardson (1926), who distinguished between classical Fickian diffusion (in which scale is not a factor) and the mixing that is characteristic of turbulent fluids such as the sea. While the nature of turbulence is extremely complex, Richardson's concept of turbulent energy passing through a series of progressively smaller eddies from the wavelength at which it is put into the fluid until it is ultimately dissipated in viscosity has continued to prove valuable in studying the mixing of marine waters (Okubo 1971). In natural systems, turbulent energy is added at a rather large scale by winds, tides, and major currents. Since none of these is usually effective in microcosm tanks or bags, some artificial means of introducing turbulent energy at smaller scale may be required to develop pelagic ecosystems that are credible experimental analogs of the "real world" (Perez et al 1977).

There are at least two aspects to the turbulence problem, one involving the actual distribution of organisms, particles, or dissolved constituents in the water – the problem of patchiness (see Steele 1974), and the other involving the metabolic or behavioral responses of organisms to water turbulence. The study of plankton patches usually concerns water masses on a scale larger than the largest microcosms yet developed (1300 m^3, 10 m dia.; see Menzel and Case 1977), and it is generally conceded that this aspect of the ecology of marine waters is not well represented in microcosm experiments. The importance of this omission is not yet known. While there have been numerous studies which have documented the response of sessile plants and animals to the special case of turbulence in flowing water (Fox et al 1935; Kerswill 1949; Whitford 1960; Jaag and Ambühl 1963; Whitford and Suchumacker 1964; McIntire 1966; Westland 1967; Nixon and Oviatt 1971), the responses of pelagic organisms to small scale turbulent energy have received much less attention.

With the exception of an older qualitative study of the morphological response of *Daphnia* to water motion by Brooks (1947), the recent work by Pasciak and Gavis (1975) on the relationship between turbulence and nutrient uptake by phytoplankton, and our own studies on marine plankton in laboratory microcosms (Perez et al 1977), the effect of water turbulence on the growth and metabolism of planktonic organisms is almost totally unknown. While this situation is largely a result of the difficulties involved in measuring turbulent energy levels in the laboratory or in the field, it may also reflect the feeling of many ecologists that the small size of plankton generally places them below the size scale at which turbulence is "felt". Both of these considerations, along with technical difficulties, have caused almost all marine microcosm studies to neglect turbulence as a factor in their experimental design. In the simplest terms, the justification appears to have been that since turbulence is difficult to measure, hard to mimic, and of unknown importance, it was reasonable to avoid the problem of deciding on how to include it in microcosms. There is a certain amount of appeal to this argument, especially since there are so many other problems to be resolved in developing a microcosm. However, the evidence in the papers cited above, as well as the experience of anyone who has tried to culture or maintain phytoplankton and zooplankton in the laboratory, suggest that turbulence is an important consideration in pelagic systems. Our earlier experiments with turbulence in marine microcosms also indicated that the scaling of mixing energy in laboratory tanks can dramatically influence the results of phytoplankton and zooplankton growth studies in the microcosms (Perez et al 1977). The argument about plankton being too small to "feel" turbulence is also questionable.

Turbulence

Following Richardson (1926), Richardson and Stommel (1948), Stommel (1949), Batchelor (1950) and others, the flow of turbulent energy from large scale motion is passed down through successively smaller eddies until it is dissipated in viscosity. Above a certain size, the energy content of eddies is solely a function of their size (k) and the rate of energy flux (ϵ) through the system. Below this critical size, defined by

$$k = \left(\frac{\nu^3}{\epsilon}\right)^{1/4} \qquad (1)$$

where ν = the kinematic viscosity
ϵ = the energy flux per unit mass
k = upper limit of the Kolmogoroff viscous zone

viscous forces become important and the energy content decays more rapidly with decreasing size as energy is dissipated. In order to give some feeling for the scale involved, it is possible to estimate k for the West Passage of Narragansett Bay using a value for the energy dissipation of 4.3×10^{13} ergs sec^{-1} (Levine and Kenyon 1975) and an approximate volume of 7.2×10^8 m^3. The result suggests that k is on the order of 0.06 cm. While this is larger than individual phytoplankton cells found in these waters (<0.01 cm), it is about the size of many of the diatom chains found in the bay (~0.05-0.1 cm) and smaller than the dominant zooplankton. Moreover, even below this size, the effect of turbulent energy persists. As the time averaged flux of energy through larger eddies is increased, the viscous shear in the Kolmogoroff zone will also increase and this increase in shear will be felt even at the very small scales seen by the plankton. Moreover, as the energy flux through larger eddies increases, the upper size limit of the Kolmogoroff zone will decrease, so that larger plankton will begin to experience direct turbulent effects.

In addition to simple mechanical effects, such as the disruption of feeding or copulation by zooplankton, this turbulent energy flux at small scale may influence the plankton (or other particles) in at least two ways. Around any given cell of size ℓ, there will exist a thin laminar boundary layer in which Fickian or molecular diffusion must be relied upon to transport dissolved gases, nutrients, waste products, etc. Since molecular diffusion is much slower than turbulent diffusion, this is often the rate limiting step in exchange processes between the cell and the surrounding medium. As the turbulent energy flux in the medium increases, however, the water just outside of the boundary layer is renewed more rapidly, with the renewal rate being proportional to:

$$R \sim \left(\frac{\epsilon}{\nu}\right)^{1/2} \tag{2}$$

This increase in renewal rate tends to maximize the concentration gradient across the laminar boundary layers and, thus, the diffusion of materials across the layer. In addition, the increase in renewal rate by turbulent velocity will also decrease the thickness of the boundary layer itself, since the boundary layer thickness is proportional to:

$$Z \sim \frac{1}{\sqrt{R}} \tag{3}$$

Again, the reduced thickness of the laminar layer will increase the exchange rate of materials between the particle and the medium.

While the cascade of turbulent energy through successively smaller eddies has been studied frequently in the sea (Okubo 1971), the emphasis in the field has generally centered on measurements of eddies larger than 10 m. The nature of the turbulent energy spectrum in small experimental ecosystems has only recently begun to receive attention (Boyce 1974, Steele et al 1977, Güst

1977). While the results of Güst's study are restricted to the specific flexible chambers and conditions of his measurements, they show quite convincingly that it is possible to obtain a small scale turbulent spectrum in a chamber that is similar to that found in the surrounding coastal waters. Unfortunately, almost all of these turbulence measurements were made in a metabolic chamber used with benthic algae rather than in plankton studies, and no biological data were included.

This paper reports the results of a number of turbulence experiments carried out at different times of the year using coupled benthic-pelagic microcosms designed as analoges of Narragansett Bay, R.I. While some of the data from experiments conducted during the spring have been reported previously (Perez et al 1977), we have now carried out identical studies during winter and summer months. In addition, we have explored in this paper a number of techniques for characterizing the turbulent mixing levels in the microcosms and compared them to the dissolution rate measurements used previously (Oviatt et al 1977, Perez et al 1977). Finally, we have attempted to carry out experiments to test our earlier conclusion that phytoplankton and zooplankton respond independently to different turbulence levels. The impression that the phytoplankton and zooplankton were not coupled in their response to turbulence was based on indirect evidence (Perez et al 1977) and we felt it desirable to test this conclusion directly by adjusting the levels of zooplankton in replicate tanks and observing whether concommitant but opposite changes would occur in the phytoplankton.

METHODS

The Microcosms

The microcosms used in these experiments have been described in detail in earlier papers (Perez et al 1977; Oviatt et al 1977 and in press). Each microcosm consisted of a 166 liter plastic tank containing 150 l of water (0.7 m deep) collected by bucket from the lower West Passage of Narragansett Bay. This area of the bay shows a well mixed water column about 8 m deep with salinity between 28-31$^{o}/oo$ throughout the year. Characteristics of the bay have been described in some detail by Kremer and Nixon (1978). The microcosms were maintained in a running sea water bath in the laboratory near field temperatures and illuminated for the appropriate natural photoperiod by Westinghouse Cool White fluorescent lights. The response of the microcosms to light input is complex and the choice of a value for any particular experiment is difficult (Nixon et al, in press). The experiments described here were carried out at 5-25 ly/day, values considerably below the average light energy found in the water column of the bay.

Each microcosm also contained an opaque plastic box ($167\ cm^2$) of intact bay sediment and associated benthos. This size produced the same sediment surface to water volume ratio as found in the bay. Water from the pelagic phase of the microcosm was moved through the box and over the sediment by vacuum pump so as not to damage the plankton. The inside walls of the tanks were cleaned regularly to prevent fouling, and organic matter settling on the bottoms of the tanks was collected and placed in the sediment boxes. In all of the turbulence experiments, it is important to note that the artificial nature of the "bottom" community isolated it from the turbulent energy of the water, except as the benthos might respond to changes in the plankton. However, even in tanks that were unstirred, the benthic box pumps provided some very gentle circulation for the pelagic community, since the flow rate used was capable of putting 150 liters of water through the box about three times each day. Additional mixing was contributed by the approximately daily wall cleaning and by the addition of 10 liters of bay water to each tank three times each week. The latter was maintained so that the microcosms functioned as open systems with a flushing rate similar to that of Narragansett Bay.

Different turbulence levels were imposed on the microcosms by leaving them unstirred except for the benthic pump and cleaning operations or by mixing them with plastic mesh paddles of $0.14\ m^2$ or $0.07\ m^2$ area. The opening size of the plastic grid in the paddles was 1.2 cm x 1.2 cm. The paddles were driven at 32 rpm by an electric motor connected to all the paddle shafts by a chain, thus producing identical rotation rates in all of the tanks. Each paddle was rotated in one direction for 30 sec., then stopped for 6 sec., then reversed for 30 sec. in a continuous cycle.

Turbulence Measurements

Vertical Eddy Diffusivity

We attempted to obtain a variety of both relative and absolute measurements of turbulent mixing in the microcosms and in Narragansett Bay. In some cases, such as the estimation of a vertical eddy diffusivity, the techniques used were conventional. A small amount (~2 ml) of Rhodamine-WT dye was dissolved in sea water (1:100) and released "instantaneously" at mid-depth in the microcosms. Near-surface and near-bottom water samples were then collected at short intervals (2-5 min.) and the concentration of dye determined fluorometrically. The rate-of-change in concentration in both sets of samples was virtually identical, indicating that the tanks were mixed uniformly up and down, and that the vertical eddy diffusivity could be estimated by

$$D_v = \frac{Z^2}{2t} \qquad (4)$$

D_v = vertical eddy diffusivity, $cm^2\ sec^{-1}$
Z = distance between relative point and measurement point (1/2 depth in this case), cm
t = time for the concentration to asymptote, sec

The average value of this parameter for Narragansett Bay has been computed by Hess (1976) using a detailed numerical hydrodynamic model.

The Horizontal Turbulent Field

Determination of the horizontal turbulent component was more difficult. At one extreme, we measured the time it took for small dye patches (0.5 ml of 1:100 dye in seawater) to disperse in the microcosms and in the bay under a range of conditions. This approach was simple, rapid, and with enough replication and a constant observer, it gave a good (low variance) relative measure of horizontal mixing rates. Unfortunately, it is also a bit subjective and qualitative and cannot be expressed directly as a standard hydrodynamic parameter.

In an attempt to overcome these limitations, we have also obtained measurements of neighbor diffusivity (Richardson, 1926; Stommel, 1948) and the flux of turbulent energy along two arbitrary perpendicular coordinates (Batchelor, 1950) using the relative motions of pairs of small floats with a range of distances separating them. The measurements were made by releasing several dozen floats and then photographing them from a fixed position at short time intervals. The size of the floats used and the length of the time interval were varied somewhat according to the scale of the turbulent eddies of interest. In the West Passage of Narragansett Bay, larger scale mixing (1-25 m) was studied using colored balloons filled with fresh water so that they floated just beneath the surface. These floats were dispersed from a small boat and photographed every few minutes from a high bridge. Smaller scale eddies (1-200 cm) were studied in the field using small (~0.5 cm) colored plastic beads that were released from the end of a pole off the stern of a small boat and photographed every few seconds using a 16 mm movie camera operated from the flying bridge of the boat. The beads and the movie camera were also used in the microcosms. In all cases, floating rods of standard length were included in each photograph to give an accurate scale.

After they were developed, the films of the floats were put through a microfiche reader for enlargement. Large numbers of pairs of floats were selected at various distances of separation to be followed from frame to frame over time. In each frame the scalar distance between the floats as well as their separation along two perpendicular vectors (x and y) was obtained. These data were then analyzed using the relationship given by Batchelor (1950).

$$\epsilon = \left[\frac{\ell x_2^2 - \ell x_1^2}{t} \Bigg/ 2t\left(1+1/3\,\frac{\ell x_1^2}{\ell o^2}\right) \quad \ell o^{2/3} \right]^{3/2} \qquad (5)$$

where ℓ_0 is the initial scalar distance between floats
ℓ_{x_1} is the initial distance between the floats projected on the x axis
ℓ_{x_2} is the distance between the floats after some time, t, projected on the x axis
ϵ is the rate of turbulent energy flux

The same operation was carried out for the 'y' vector which should yield a similar value if the turbulent field is isotropic. Unfortunately, it is extremely difficult to extend the analysis to a third dimension and it is not practical to use the method to explore the horizontal turbulent field below the surface.

Additional Relative Turbulence Measures

As discussed earlier, the flux of turbulent energy influences the rate at which materials may be exchanged across a laminar boundary layer. This suggests that the dissolution rate of a solid substance placed in the water may, at least to some degree, be a function of the turbulent energy of the fluid. The importance of the turbulent effect should be greatest for materials that are near saturation in sea water. After some exploration, we have found that the mineral gypsum ($CaSO_4$) is particularly well suited for this purpose. It is easily obtained, inexpensive, and a large number of uniform pieces can be cut from a single rough block. The dissolution rate is influenced somewhat by temperature and salinity, but these relationships are easily established in the laboratory in order to compare measurements made under different conditions. Since the rate of weight loss is also a function of size, we have found it best to use standard pieces of gypsum measuring ~2.5 x 1.8 x 0.7 cm with an initial weight of about 6-8 gms. Blocks of this size are suitable for making measurements of weight loss over periods ranging from about 6-24 hrs. Replication appears to be quite good and duplicate blocks are hung off a fixed or free floating line to obtain a vertical profile of dissolution rate. Unfortunately, however, the $CaSO_4$ dissolution rate may provide only a relative measure of turbulent energy and it is not clear if it is possible to relate it to any more conventional physical measurements. It is also not clear how one might separate vertical and horizontal components of mixing using the blocks.

An additional indirect estimate of mixing in the microcosms and in the field was obtained by measuring the diffusion coefficient for oxygen across the air-water interface. The measurements were made using a small floating plastic dome from which virtually all of the oxygen was displaced by nitrogen gas. The partial pressure of oxygen in the dome and in the water was then monitored over time and the flux of oxygen from the water into the dome calculated. Since the flux is a product of the gradient in partial pressure and the diffusion coefficient, it was possible to obtain the coefficient from such a data set. Measurements with domes containing turbulent or still air have confirmed that the diffusion coefficient is largely a function of turbulence in the liquid phase, and that the effect of wind is felt through its influence on water mixing.

RESULTS

Turbulence Levels Obtained

The results of the various turbulence measurements lead us to be particularly sympathetic to Okubo's lament that "diffusion is confusion." In spite of, or perhaps because of the fact that a large number of floating pair observations were made in Narragansett Bay and in the microcosms, there was a very large amount of scatter in these data. As a result, the calculation of neighbor diffusivity (F) and energy flux (ϵ) was subject to a large uncertainty and there is some question about how meaningful the numbers may be. While the values of F tended to decrease approximately according to the 4/3 law (Richardson 1926; Stommel 1948), it also appeared that ϵ had a tendency to fall off with size. The latter result is disturbing since the theoretical framework for the computation suggests that ϵ should be constant at steadystate in the inertial range between the size at which energy is put into the system and the viscous zone in which it is dissipated.

Functional regressions (Ricker 1973) relating F to scale are given below. In general, both the neighbor diffusivity and the energy flux indicated that the turbulence levels in the microcosms with one paddle were appreciably higher than found in the bay or in the mocrocosms with one half paddle and no paddle (Table 25-1). The F Values did not show a great difference between the half paddle microcosms and the bay and the very large variance associated with the calculation of ϵ in the half paddle tanks made it difficult to see any clear differences in terms of energy flux.

Table 25-1. Estimates of Turbulent Energy Dissipation Rates in the Experimental Microcosms and in Some Natural Marine Waters

	ϵ, $cm^2\ sec^{-3}$
Microcosms with one paddle	1.44
Microcosms with half paddle	0.03
Microcosms with no paddle	$\rightarrow 0$
Narragansett Bay, West Passage measured at different times	0.05, 0.07, 0.11, 0.17, 1.0
Narragansett Bay, West Passage estimated from tidal currents, (Levine and Kenyon, 1975)	0.21
Irish Sea, estimated from tidal currents (Taylor, 1919)	0.08
N.W. Pacific coastal water, U.S.A. surface 30m, calcualted from changes in microstructure (P.W. Nasmyth in Gregg 1973)	0.02
Open sea, surface mixed layer (50 m) (Gregg 1973)	0.002

$$\begin{aligned} &\text{Functional Regressions for } F,\ cm^2\ sec^{-1} \\ &\text{West Passage } F = 0.007\ \ell 1.89 \\ &\text{Microcosms with one paddle } F = 0.108\ \ell 1.38 \\ &\text{with half paddle } F = 0.013\ \ell 1.73 \end{aligned} \qquad (6)$$

The simpler measurements of horizontal dye patch dispersion suggested that while the one paddle microcosms were considerably more turbulent than those with half a paddle, both were more rapidly mixed than the bay (Table 25-2). This same trend with respect to differences between the whole paddle and half paddle microcosms was also shown by the weight loss of gypsum blocks and the gas exchange measurements (Table 25-2). However, both of these parameters indicated substantially higher mixing rates in the bay than in any of

Table 25-2. Relative Turbulence Measurements in the Experimental Microcosms and in Narragansett Bay

Vertical Eddy Diffusivity

	K, $cm^2 sec^{-1}$
Microcosms with one paddle	3.6 - 3.8
Microcosms with half paddle	2.2 - 3.8
Microcosms with no paddle	$\rightarrow 0$
Narragansett Bay, mean for the West Passage (Hess 1976)	5

Rates of Horizontal Dye Patch Dispersion

	Time to Disperse, sec
Microcosms with one paddle (N=7)	5.4 ± 1.2
Microcosms with half paddle (N=7)	8.4 ± 1.2
Microcosms with no paddle (N=1)	$\gg 900$
Narragansett Bay, West Passage	
day 1 (N=10)	18.0 ± 5.4
day 2 (N=6)	13.4 ± 1.9
day 3 (N=6)	17.5 ± 2.5

$CaSO_4$ Dissolution Rate

	Weight Loss, % hr^{-1}
Microcosms with one paddle	1.83 ± 0.20
Microcosms with half paddle	0.73 ± 0.36
Microcosms with no paddle	0.27 ± 0.05
Narragansett Bay, West Passage, mean for vertical profiles	3.30 ± 0.60 4.39 ± 1.10

O_2 Diffusion Coefficient at the Air-Water Interface

	K, $\mu M\ m^{-2} hr^{-1} atm^{-1}$
Microcosms with one paddle	27
Microcosms with half paddle	8
Microcosms with no paddle	2
Narragansett Bay, West Passage	
calm day	30
windy day	137
windy day	125

the microcosms. To some extent this may reflect the fact that the floating pair measurements and dye patches respond to the horizontal turbulent field and the gypsum and gas exchange are also influenced by vertical motion. The rotating plastic paddles appeared to add a lot of horizontal mixing energy to the tanks, but the vertical eddy diffusivity in the microcosms was lower than Hess (1976) calculated for Narragansett Bay (Table 25-1).

While none of these measurements allows us to make a very convincing absolute comparison of turbulent energy in the microcosms with that of the bay, it does seem clear that the full paddle, half paddle, no paddle configuration provided quite different turbulent water regimes in the microcosms. Since the input of turbulent energy to Narragansett Bay must vary considerably during the tidal cycle and from day-to-day according to the winds, it seems reasonable that the natural pelagic community may well experience all of the turbulent conditions used in the microcosms. For comparative purposes, it is interesting to note that all of the methods used for measuring turbulence except the determination of neighbor diffusivity and energy flux (ϵ) indicated that even the full paddle configuration was low relative to the bay.

Response of the Plankton

The first turbulence experiment was carried out during the month of April when water temperatures in the microcosms ranged from 8 to 12°C. The standing crop of phytoplankton as indicated by chl a increased dramatically in the one paddle and half paddle treatments compared with the unstirred tanks (Figure 25-1). A number of cursory analyses of water samples did not indicate that there were any major shifts in species composition in the different tanks. However, there were also marked and significant differences (Perez et al 1977) among treatments in the numbers of *Acartia clausi*, the dominant zooplankton in the microcosms and in the bay (Figure 25-2). While the rapid increase in phytoplankton in the one paddle tanks began almost immediately, *Acartia* nauplii did not really start to decline until after 10 days. In fact, a portion of the decline in nauplii between 10 and 16 days was simply due to growth of the animals into juveniles (Figure 25-2). An analysis of covariance was performed to establish whether the changes in phytoplankton density could be attributed to changes in zooplankton density the covariate, total grazers was found to be non-significant. This meant that the inverse relationship expressed by zooplankton and phytoplankton to water turbulence was due to a direct pattern than the indirect effect of water turbulence. In fact, an analysis of covariance on the mean algal standing crop during the experiment indicated that interactions with the total numbers of grazers in the microcosms (the covariate) was not significant (Perez et al 1977). It is possible, however, that the zooplankton present did not feed as effectively in the more turbulent

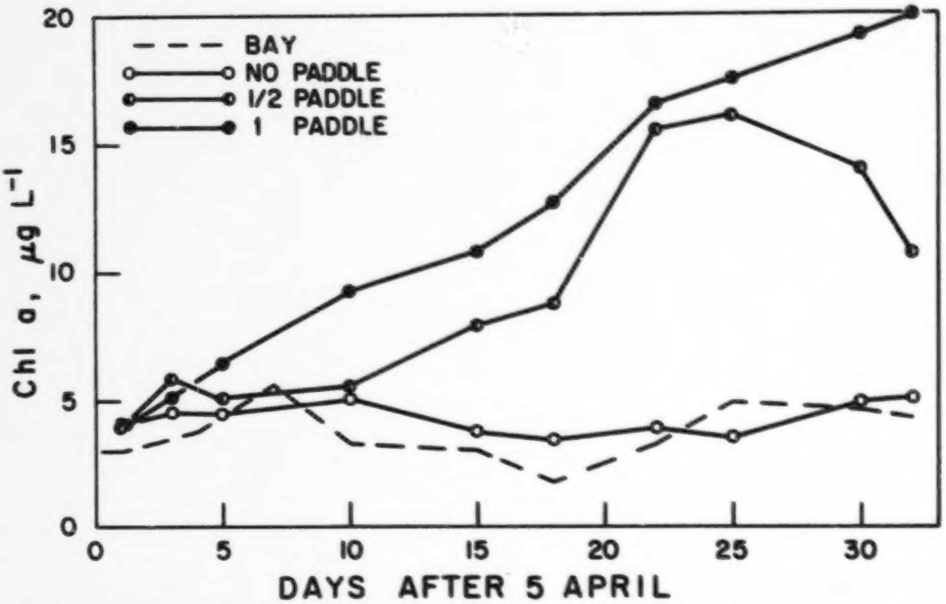

Figure 25-1. Chlorophyll Concentrations (In Vivo Fluorescence) in the Microcosms and in the Lower West Passage of Narragansett Bay During the First Turbulence Experiment Begun April 5, 1976.

NOTE: Data points are the mean of duplicate tanks.

tanks. It is striking that the zooplankton standing crop with one paddle was very similar to that found during the same period in the bay, while the phytoplankton populations in those tanks reached levels 3 times greater than found in the bay with similar zooplankton numbers. Conversely, the low and relatively constant phytoplankton standing crops in the unstirred microcosms were almost identical to that found in the bay, but much larger zooplankton populations were sustained, at least for 15 days, in the microcosms. It may be that the plankton in the microcosms escaped a significant grazing and/or predation pressure that was important in setting the standing crop maintained in the field. This experiment was repeated during May with virtually the same results.

The next turbulence experiment was not begun until December, when water temperatures ranged from 1 to 6°C and the standing crops of phytoplankton were low. The experiment was designed to explore not only the effect of turbulence, but also the interactions of turbulence with light and nutrient enrichment. Again, the standing crop of phytoplankton was significantly (0.05 level) higher in tanks mixed with a full paddle, though the effect was not as dramatic as in the earlier runs (Figure 25-3). The response to light was not significant at the 0.05 level but was significant at the 0.10 level. The response to turbulence was highly significant (greater than the 0.01 level) (Figure 25-4).

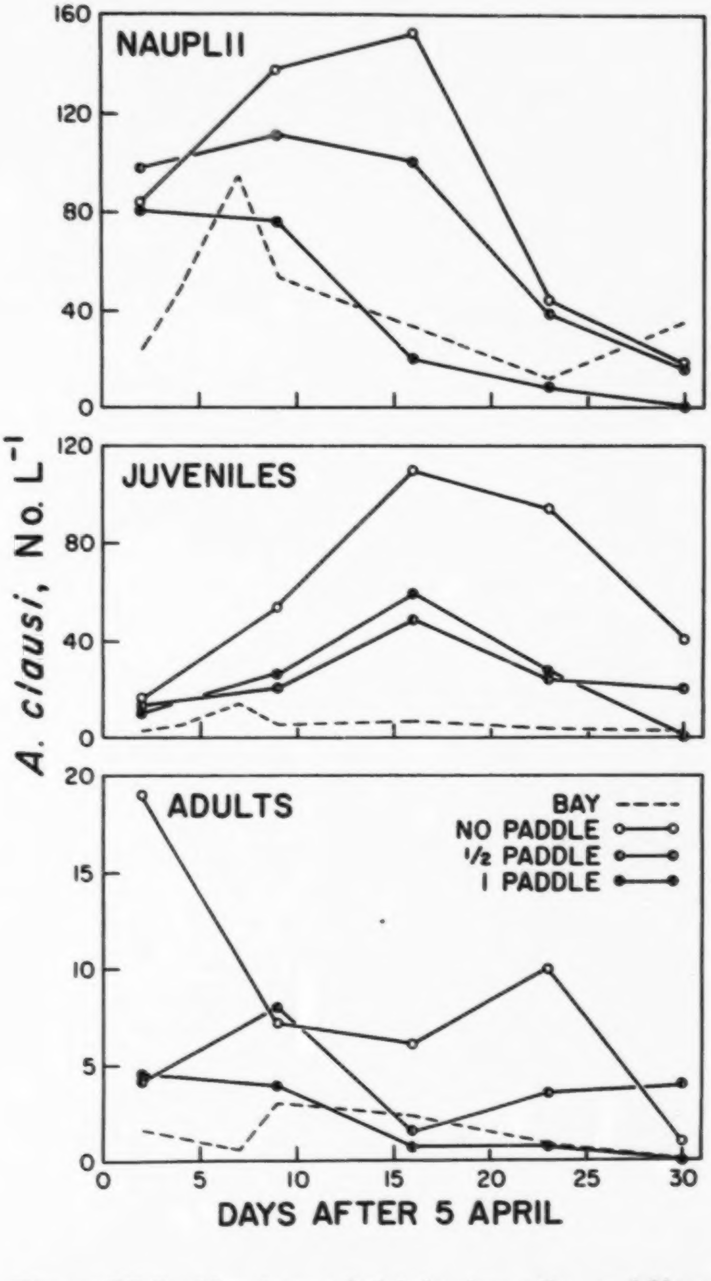

Figure 25-2. Numbers of Adult, Juvenile, and Nauplii Acartia clausi in the Microcosms and in the Lower West Passage of Narragansett Bay during the First Turbulence Experiment.

NOTE: Data points are the mean of duplicate tanks.

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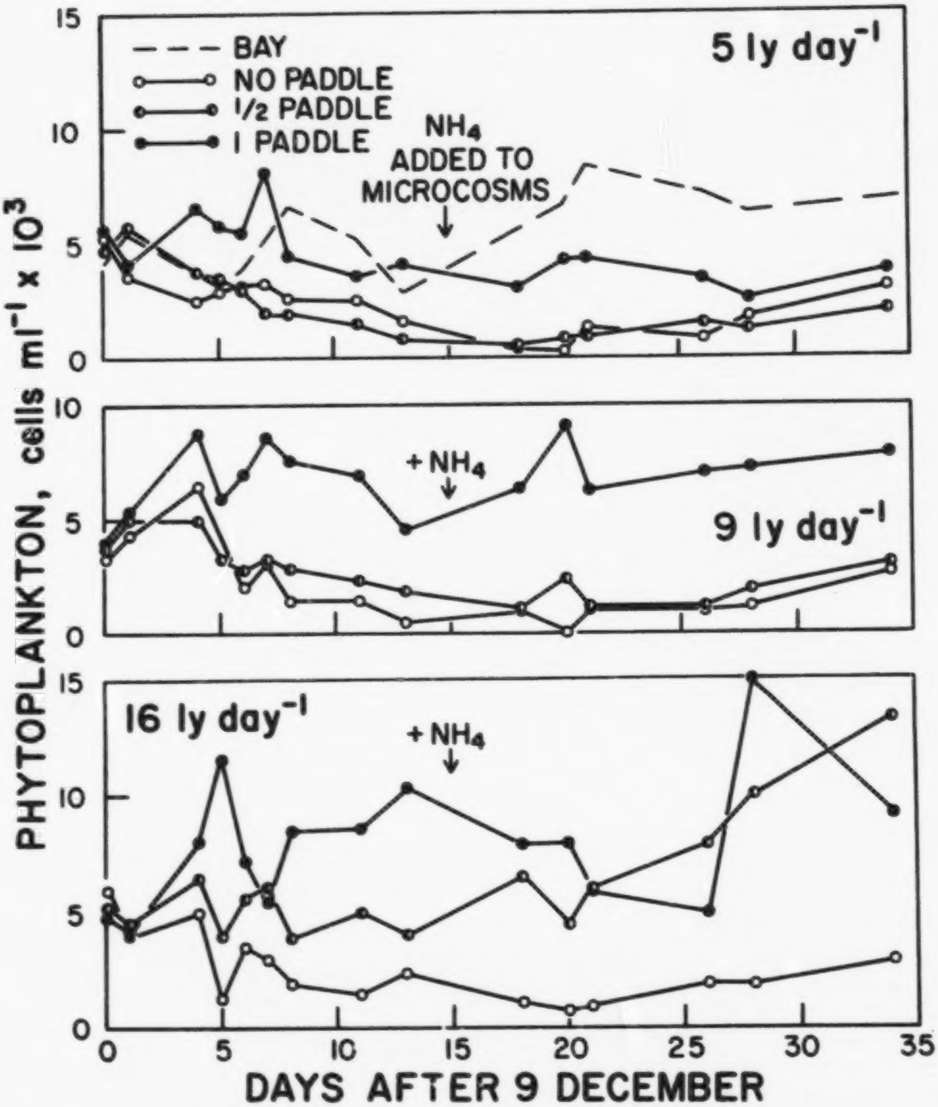

Figure 25-3. Phytoplankton Cell Counts in the Microcosms and in the Lower West Passage of Narragansett Bay During the Turbulence-Light Interaction Experiment Begun December 9, 1976.

NOTE: Data points are the mean of duplicate tanks.

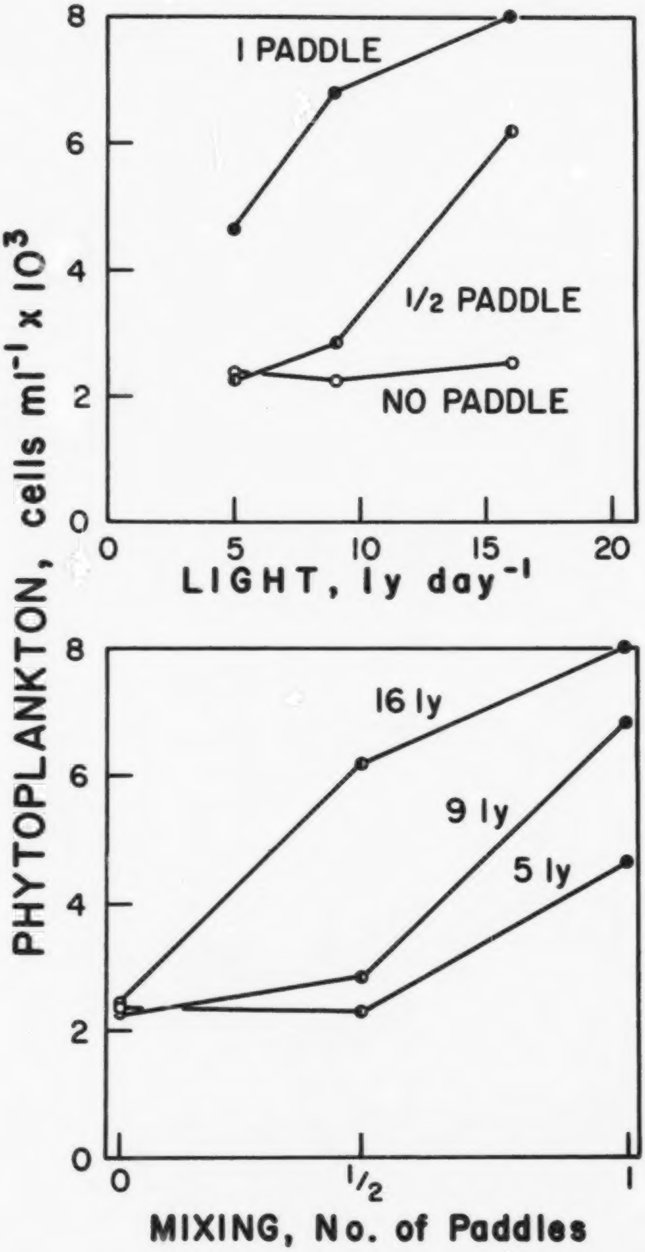

Figure 25-4. Average Phytoplankton Population Levels During the 34 Day Turbulence-Light Experiment.

NOTE: Data points are the mean of duplicate tanks.

The interaction of light and turbulence was not significant nor was the effect of ammonia enrichment. However the ammonia addition brought the concentration in the tanks from ~3μM to ~6μM, so that the plankton were never seriously nutrient limited. It was also interesting that there was no response of the phytoplankton in the unmixed microcosms to increased light, while there was a clear increase in the stirred tank populations with higher light levels (Figure 25-4). The numbers of zooplankton, again dominated by *A. clausi*, were very low throughout the experiment (Nauplii ≈10/L; juveniles ≈5/L) and no dramatic differences among treatments developed. However, the mean numbers of nauplii and juveniles observed during the experiment were higher in the tanks with no paddle and lowest in the tanks with one paddle. Analysis of the data showed that this difference in the means was statistically significant (α:0.05) and that there was no significant interaction of nauplii, juveniles, or adults with light intensity. There was no statistically significant difference in the mean number of adults in the different turbulence levels.

In order to find out if turbulence had a direct stimulating effect on phytoplankton, two experiments were carried out during January and February in which an attempt was made to remove zooplankton from some of the microcosms by filtering the water through a #20 (80 μ) net. This was effective in reducing the zooplankton levels by about 70 percent in the first experiment and by about 90 percent in the second. In addition, light levels were increased from 6 ly/day during the January run to 16 ly/day in February and ammonia was added to all tanks at the start of the second experiment in an attempt to stimulate vigorous phytoplankton growth. Temperatures ranged from 0-0.5°C during the first experiment and from 0-3°C during the second.

The results of the first experiment showed no significant effect of turbulence on the numbers of phytoplankton or zooplankton in the microcosms (Figures 25-5 and 25-6). The lack of turbulence effect on the phytoplankton was observed in tanks with and essentially without zooplankton (Figure 25-5). It is interesting to note that the variation in zooplankton numbers by a factor of about 3.5 had no significant effect on the levels of phytoplankton, probably due to low temperatures and therefore reduced grazing rates.

When the experiment was repeated a month later with higher light and nutrients, a phytoplankton bloom was produced during the first week in all of the microcosms (Figure 25-7). During this period there did not appear to be any effect of the turbulence on phytoplankton growth either with or without zooplankton. Moreover, the grazing pressure of the small number of *A. clausi* in the unfiltered water (~15 animals/L) at these low temperatures had little or no effect on the bloom. However, the bloom declined much more slowly in the unstirred microcosms, so that after 10-15 days the standing crops in the

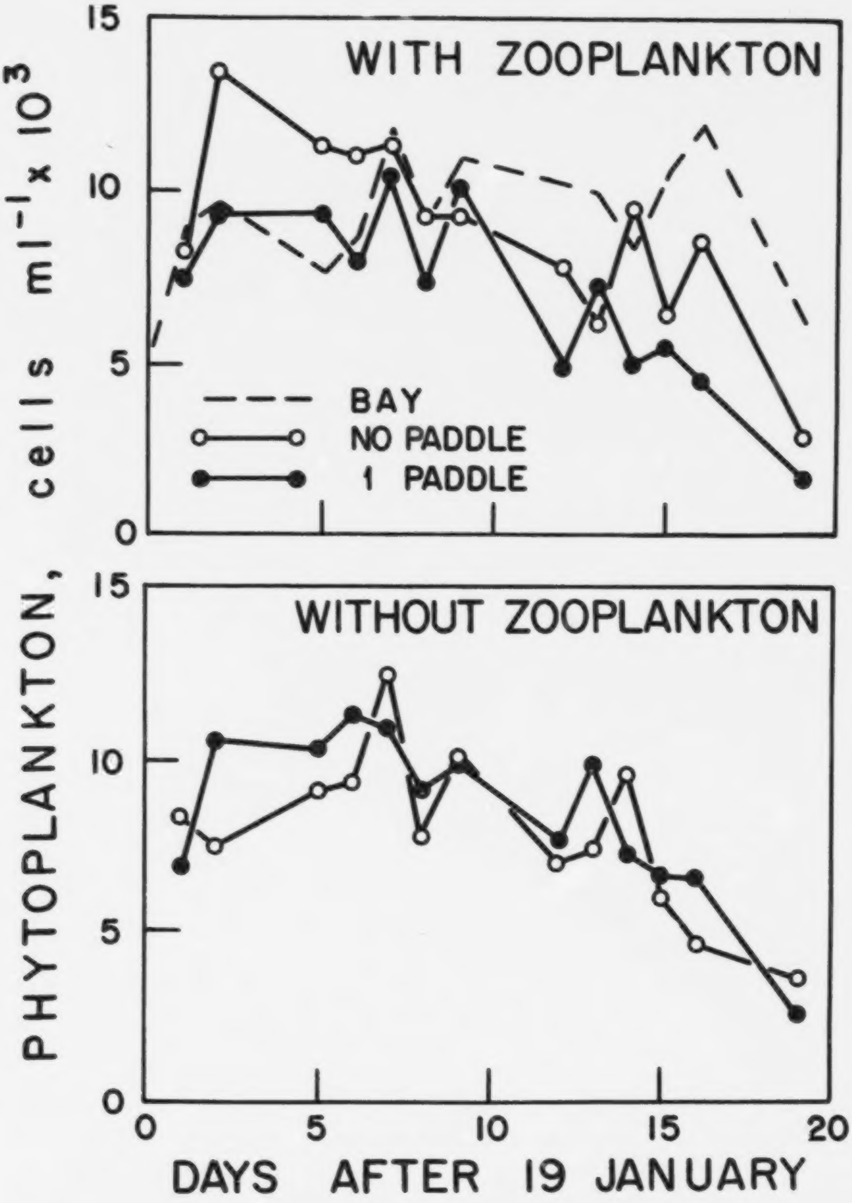

Figure 25-5. Phytoplankton Cell Counts in the Microcosms and in the Lower West Passage of Narragansett Bay During the January 1977 Turbulence Experiment.

NOTE: Data points are the mean of duplicate tanks.

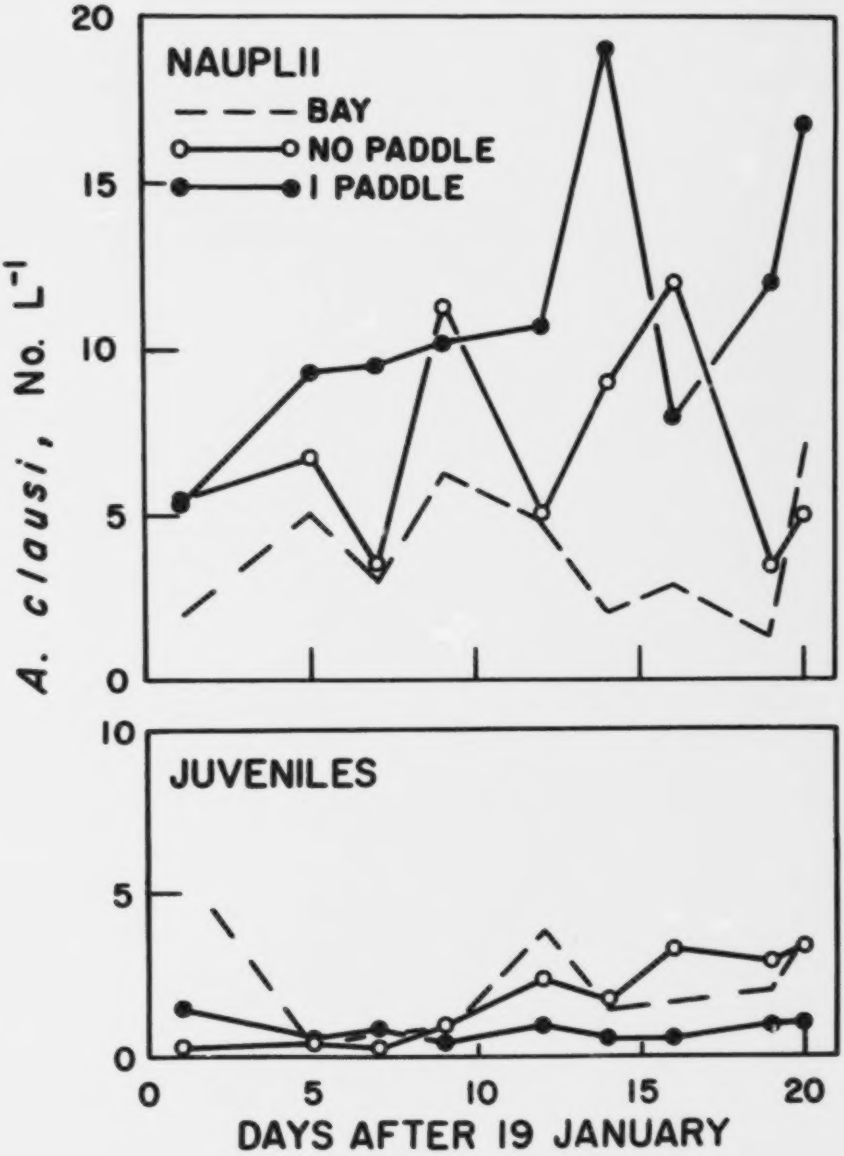

Figure 25-6. Numbers of Acartia clausi nauplii and Juveniles in the Unfiltered Microcosms and in the Lower West Passage of Narragansett Bay During the January Turbulence Experiment.

NOTE: Data points are the mean of duplicate tanks.

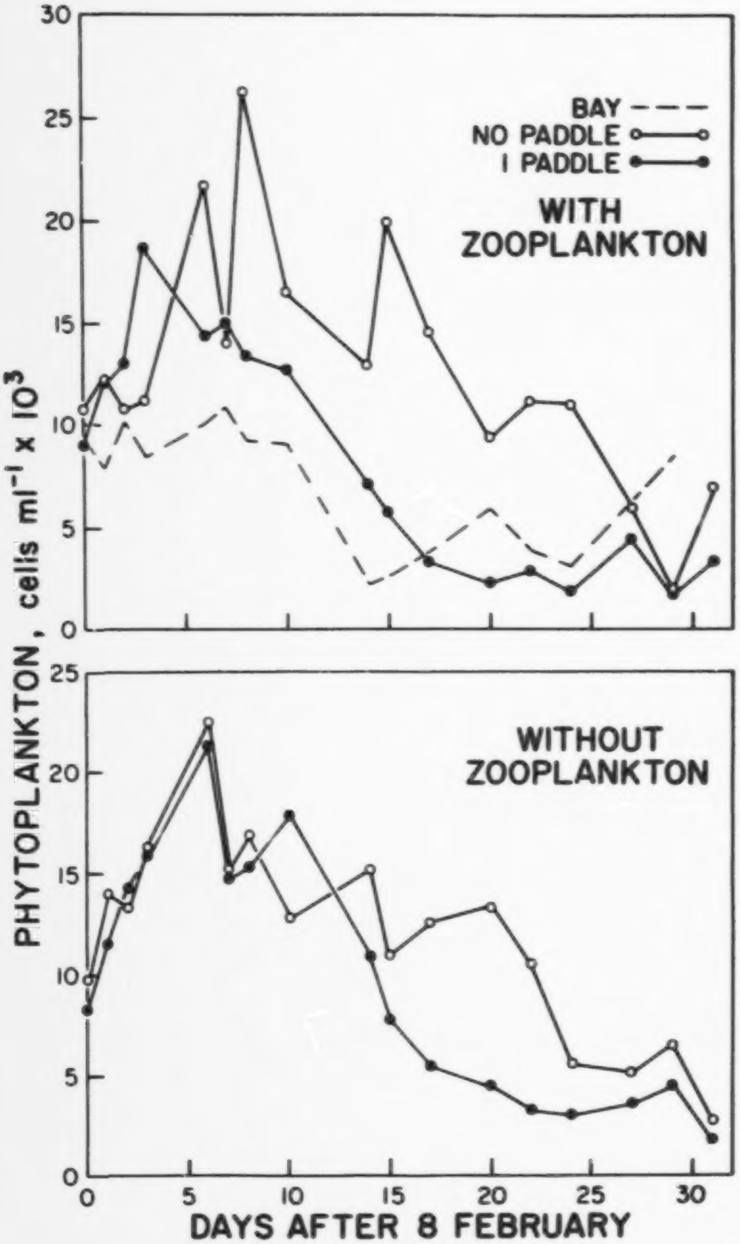

Figure 25-7. Phytoplankton Cell Counts in the Microcosms and in the Lower West Passage of Narragansett Bay During the February 1977 Turbulence Experiment.

NOTE: Data points are the mean of duplicate tanks.

turbulent tanks were markedly lower both with and without zooplankton (Figure 25-7). Statistical analysis of the mean numbers of cells in each treatment during the experiment showed that the average standing crop of phytoplankton was significantly higher ($a = 0.05$) in the unstirred tanks. Again, this is clearly the reverse of the pattern found in the first three experiments, but repeats the trend suggested by the January run (Figure 25-5). There were no significant differences in the new numbers of zooplankton between the two turbulence levels during this experiment, with both showing small populations that fluctuated between about 5-15 animals per liter. This is the first experiment in which the phytoplankton showed a significant response to turbulence (albeit opposite to that found previously) but zooplankton numbers did not. Again, it is interesting to note that an almost 9 fold increase in zooplankton numbers did not result in any statistically significant decline in the numbers of phytoplankton.

We attempted to repeat the zooplankton removal experiment during July of the following summer with water temperatures between 19-20.5°C. However, the hatching and development rate of zooplankton eggs and nauplii is so rapid at the higher temperatures that it was virtually impossible to reduce the numbers of zooplankton very much by the filtration method used. Nevertheless, the results were interesting. The pattern found in the first three experiments emerged once again, with phytoplankton growth clearly enhanced by the turbulent mixing and zooplankton surpressed (Figures 25-8 and 25-9).

A final experiment was carried out during August in which the interaction of turbulence and water turnover rate in the microcosms was explored. Water temperatures varied between 19-21°C. While there was no significant effect of turnover rate on the plankton, the same statistically significant stimulation of phytoplankton growth was found in the stirred microcosms where zooplankton significantly declined by a factor of 2-3 (Figure 25-10). The ten-fold increase in phytoplankton associated with somewhat more than a halving of the zooplankton in the turbulent microcosms may reflect the tight coupling of these two compartments that has been suggested in numerical simulations of the summer plankton (Kremer and Nixon 1978). This result contrasts with our earlier experiments carried out at lower temperatures in which significant reductions in zooplankton numbers had no significant effect on the mean phytoplankton standing crop.

DISCUSSION

The Importance of Turbulence

It seems clear that the presence or absence of turbulent mixing in the microcosms had a significant influence on the abundance of phytoplankton

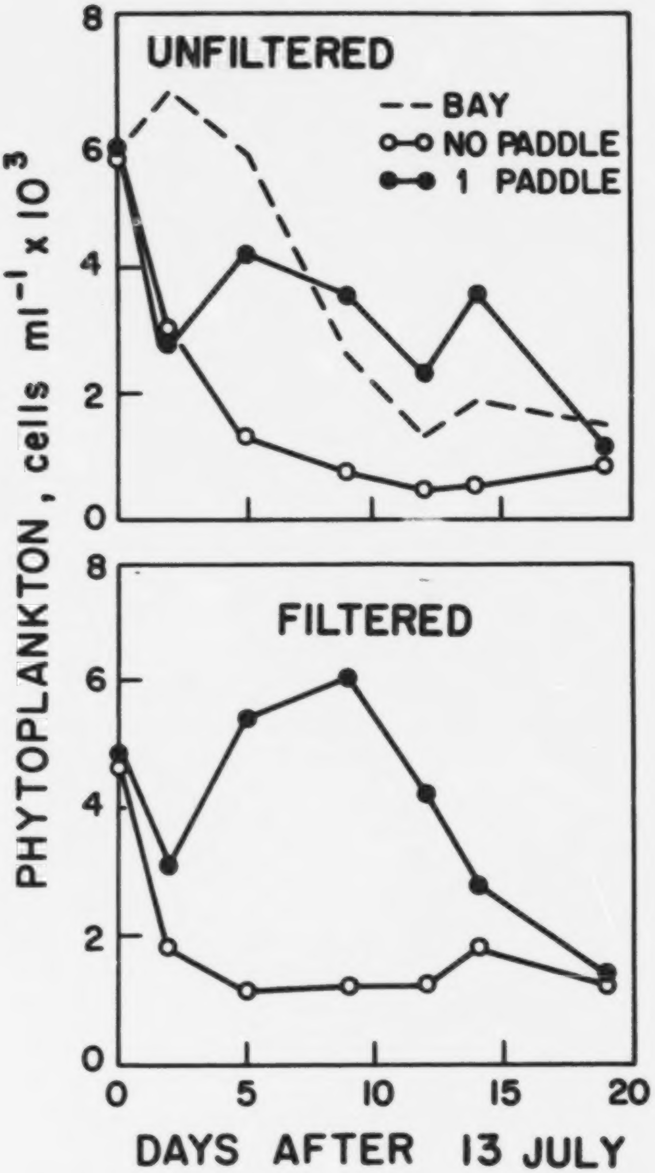

Figure 25-8. Phytoplankton Cell Counts in the Microcosms and in the Lower West Passage of Narragansett Bay During the July 1977 Turbulence Experiment.

NOTE: Zooplankton numbers in the filtered tanks (80μ net) were only slightly lower than in the unfiltered (see Figure 25-9). Data points are the means of duplicate tanks.

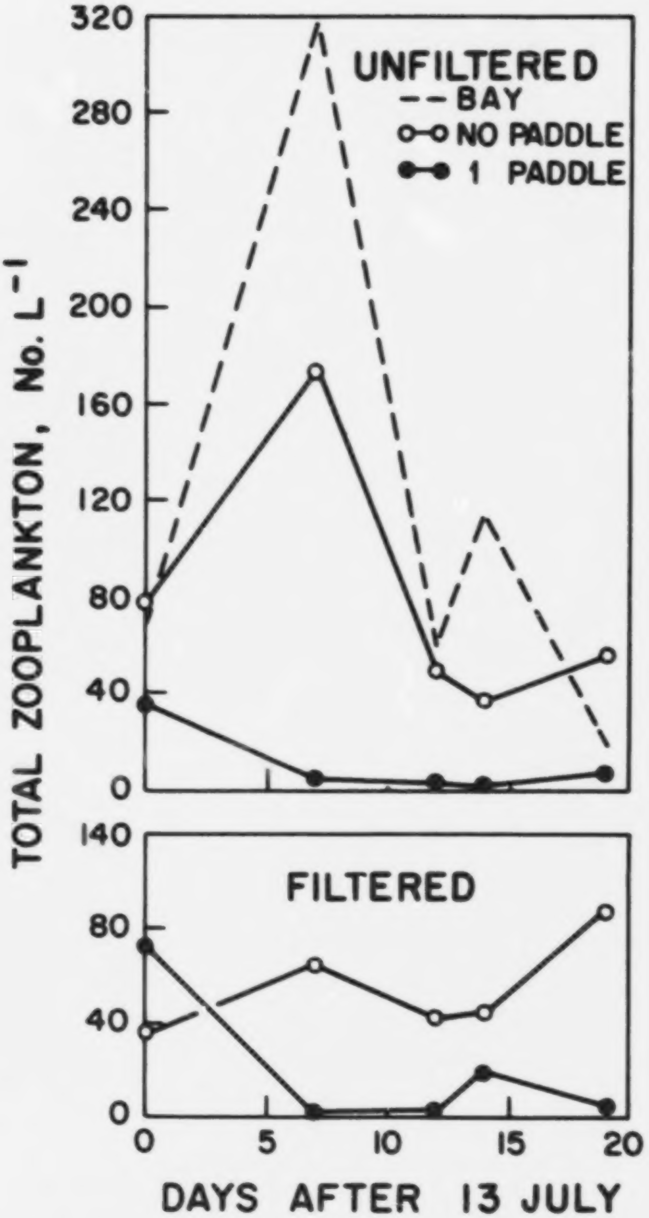

Figure 25-9. Total Zooplankton Counts in the Microcosms and in the Lower West Passage of Narragansett Bay During the July 1977 Turbulence Experiment.

NOTE: Data points are the mean of duplicate tanks.

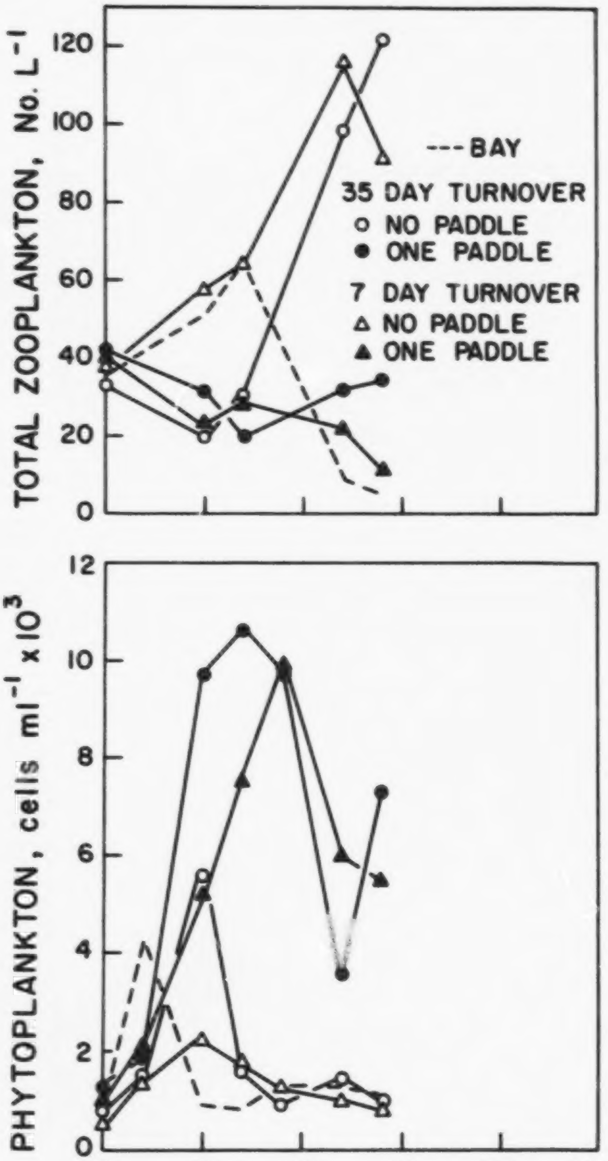

Figure 25-10. Counts of Phytoplankton and Total Zooplankton in the Microcosms and in the Lower West Passage of Narragansett Bay During the August 1977 Turbulence Experiment.

NOTE: Data points are the mean of duplicate tanks.

and zooplankton during the warmer months. Unfortunately, the results of the experiments do not make it clear if the turbulence effect is felt directly by both populations or if the enhancement of phytoplankton growth is the result of lower zooplankton grazing pressure in the more turbulent tanks. The lack of a significant turbulence effect on phytoplankton during the colder months may result from the fact that the phytoplankton and zooplankton virtually do not interact at low temperatures when feeding rates and excretion approach zero (Heinle and Vargo, 1978). During the warmer months there is evidence from some of our other microcosm experiments that the zooplankton are more effective at cropping down phytoplankton than a 60 percent artificial level of cropping imposed biweekly (Oviatt et al in press). In some cases, such as the April run, it appeared that the lower grazing pressure might be due to less effective zooplankton feeding as well as to a higher zooplankton mortality in the well mixed microcosms. At this point, however, it is still not clear if this increased zooplankton mortality was the result of a real physiological or behavioral response to the turbulent field or if it was a simple mechanical artifact resulting from the manner in which turbulence was generated.

Not only is the physical basis of turbulence confusing, but, at least at this point, so are its ecological consequences. The experiments described here are among the first ever reported on this problem, and it is not surprising that so much remains obscure. The results demonstrate the potential significance of turbulence as an ecological factor in pelagic systems and illustrate the importance of carrying out relatively long term (15-30 day) experiments at different times of the year, or at least at different temperatures, when studying the problem. It is also important to explore different ways of generating turbulence as well as the effects of its intensity in experimental ecosystems.

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